PHYSICO-CHEMICAL STUDIES

on the

INTERACTION OF METALS

with

PROTEINS, AMINO ACIDS AND RELATED COMPOUNDS

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A C K N O W L E D G E M E N T

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C O N T E N T S

G E N E R A L I N T R O D U C T I O N

GENERAL INTRODUCTION

Investigations on proteins present a number of complexities which have been attributed to factors, like uncertainity in molecular weight, variation in chemical behaviour depending on the nature of origion, purity and great sensitivity to denaturation. It is, therefore, not surprising that workers in this particular field of study are confronted with the theoretical and practical difficulties of almost unresolvable magnitude. But according to Edsall[1], "These considerations should not discourage the investigator of proteins from proceeding with his work". On the other hand "they should cause him to proceed in constant awareness of the delicacy and complexity of the structures with which he deals, of the imperfections and dangers of all fractionation procedures, and of the frequent need for revising his methods and his conclusions as better methods become available". The validity of the above mentioned statement is amply borne out by considering protein interactions, especially those with metal ions- a type which besides being of fundamental importance in explaining complicated metabolic processes[2-11] in living organisms offer fresh avenues for further work both for physical chemist and technical analyst. Truly speaking, the fundamental and applied strides which have been achieved during the course of last ten to fifteen years, may be taken as the direct outcome of the judicious use of different

physico-chemical techniques. In other words, a brief survey of these techniques may be considered as a means of tracing the history of the development of the knowledge of metal-protein interactions.

A large number of physico-chemical methods, viz., uttracentrifugation[12,13], ultrafiltration[14-18] Magnetic susceptibility[19-21], light Scattering[22-24], solubility[25], Viscosity[26], Precipitation[27-29], Migration in Electrical field[30-32], Equilibrium dialysis, Electrometry, Photometry etc., have been extensively employed both for qualitative and quantitative studies. Of these methods, equilibrium dialysis[33-43], potentiometry and pH-metry[44-49], Polarography[49,50-5] Spectrophotometry[39-41,59-67] and electrophoretic mobility[68-69] have provided means of ascertaining the mode of binding of the metals to the protein and in achieving greater success in the solution of the quantitative aspect of the problem. The results of the investigations on the interaction of metals or, as in some cases, their hydrous oxide sols with protein and amino acids, described in the thesis, are based on the use of some of the latter techniques. As would later be seen, their use has supplied much useful and, in many cases, new information on the problem of metal-protein interaction as a whole.

A fitting prelude to any survey of metal-protein interaction would be a reference to the problem of hydrogen inn equilibria of proteins. These studies are indispensible for the determination and characterisation of different

ionisable sites acting as donor groups for the coordination of metal ions in the proteins. Theoretical interpretation of the problem, first sought by Linderstrom-Lang[70-71] on the basis of Debye-Hukel theory was further extended by Cannan et al[72-73], kirkwood[74] and Scatchard[75]. Extensive studies on the problem were carried out by Tanford[76-86] who was able to determine the number of different reactive groups as well as their pK values, using the equation[47].

$$\log \frac{V_i}{n_i - V_i} = (pK_{int}) - 0.868\ ZW$$

where n_i is the number of ionisable groups with pK equal to (pK_{int}), V_i the number of groups ionised, Z the net charge on protein molecule and $W = \frac{N e^2}{2DRT}\left(\frac{1}{b} \quad \frac{k}{ka}\right)$ where b is the radius of protein, a the radius of exclusion and N, e, D, T and R have the usual significance in the Debye/Huckel theory. If the Linderstrom-Lang's model of protein is adequate, a plot of pH-log $\frac{V_i}{n_i - V_i}$ against Z or more conveniently[87] h, the number of protons bound per protein molecule, would be linear with the intercept (at h = 0) equal to (pK_{int}). The value of electrostatic factor W can be computed from the slope of the straight line. Hence it is found that hydrogen ion titration curves are enough to provide the necessary information regarding the electrochemical nature of the protein molecule with an additional knowledge of number of ionisable groups.

Theory of Multiple Equilibria[88]

When proteins combine reversibly with small molecules or ions the usual laws of equilibrium govern the reaction. Since proteins in general offer a large number of reactive sites for metal ion binding, therefore, the successive reactions in the multiple equilibria of a metal ion, M, with a protein molecule P, having n sites for combination may be represented by the equations.

$$P - M \qquad PM$$
$$PM - M \qquad PM_2$$
$$PM_2 - M \qquad PM_3$$
$$- - - - - - - - - - - - - - - - -$$
$$PM_{n-1} - M \qquad PM_n$$

The individual formation constants may be given according to law of mass action

$$K_1 = \frac{(PM)}{(P)\ (M)}$$

$$K_2 = \frac{(PM_2)}{(PM)\ (M)}$$

$$K_3 = \frac{(PM3)}{(PM_2)(M)}$$

$$. . . . . .$$

$$K_n = \frac{(PM_n)}{(PM_{n-1})\ (M)}$$

Now if $V = \frac{\text{Moles of bound M}}{\text{Moles of total Protein}}$ it can be shown

that $$V = \frac{(PM) - 2\ (PM_2) \quad - . . . . . n(PM_n)}{(P) - (PM) - . . . . . .(PM_n)} \quad . . . (i)$$

or $$V = \frac{K_1(M) - 2K_1K_2(M)^2 - . . . . . n\ K_1K_2 . . . K_n(M)^n}{1 - K_1(M) - K_1K_2(M)^2 - . . . . K_1K_2 . . . K\ (M)^n} \ldots (ii)$$

If each site, uninfluenced by its neighbouring sites has the same affinity for M, then $K_1 \ldots K_n$ are not independent but bear a relationship with each other and to a single intrinsic constant K.

$$K_2 = \frac{n - i - 1}{i} K \quad \text{where } i = 1,2 \ldots n$$

equation 2 therefore reducesto

$$V = \frac{nK(M)}{1-K(M)} \quad \ldots\ldots\ldots \quad \text{(iii)}$$

But when an ion is bound to protein, it tends to reduce the affinity of the protein for the second on coming ion due to electrostatic repulsion and hence equation 2 would no longer be valid. This electrostatic factor may be introduced into equation (3) by an approximation[70], fairly accurate for the magnitudes of the electrostatic effects commonly ecountered in ion-protein interaction and thus the equation (3) becomes

$$V = \frac{nKe^{-2z^2W}(M)}{1 - Ke^{-2Z2W}(M)} \quad \ldots\ldots\ldots \text{(iv)}$$

where Z is the charge of the metal ion and W a quantity related to the electrostatic work required to bring the ion M to the surface of the protein molecule, and for a spherical model it is given by the expression

$$W = \frac{Ne^2}{2DRT} \left(\frac{1}{b} \quad \frac{k}{1-k_a}\right)$$

WHERE b is the radius of protein, a_{the} radius of exclusion and N, e, D, R and T have the usual significance in the Debye-Huckel theory. In a competitive interaction, the

two cations, say M and N may compete for the same site. Neglecting electrostatic interaction factor, the equation for a single set of sites reduces to

$$\bar{V}_M = \frac{n K_M (M)}{1 + K_M (M) + K_N (N)}$$

Metal - Protein Systems

The amount of work done and the experimental data accumulated on metal protein systems is so large that it is difficult even to give a brief review of the work done in recent years. Generally speaking the studies carried out so far, may be divided under the following heads (i) the evaluation or determination of the intrinsic association constant, (ii) a comparison of these constants with the formation constants of simple ligands having the same donor groups as the proteins; (iii) Experiments performed to show the absence or presence of the phenomenon of chelation and (iv) the study of systems involving 'sluggish equilibrium' e.g., formation of poly-nuclear complexes[89] of iron, chromium and aluminium. The results reveal that the interaction of mercury, copper, silver, zinc, lead and cadmium with proteins have been the most extensively investigated. These metals are strongly bound to different reactive sites on the protein molecule, being preferentially bound to - SH group at lower concentration Moreover, they generally form stronger bonds with nitrogen than with oxygen atom, and hence their interaction with

imidazole ε-amino and guanidino groups are more significant.

Interaction with -SH group of the protein have been mainly carried out with mercury[22,90-91], or its mono-alkyl derivatives[10]. The results with serum albumin indicate, that one metal ion is bound with two protein molecules forming a dimer. Higher concentration of the metal ion is found to oppose the formation of dimer and so also the excess of halide ions. Methyl mercuryl salts react quantitatively with sulphhydryl groups with $pK = 4.5$[22] (also see amperometric studies by Kolthoff and Harris[92] for the estimation of -SH group in mercaptans). The sulphhydryl enzymes viz., enolase[93], lactic acid dehydrogenase[94] and pepsin[95] form mercury complexes at the cost of their enzymatic activity which can be restored by cystine. One of the most interesting reaction of proteins with mercury is that of lysozyme known to devoid of -SH group. Smith and coworkers[96] have shown that three mercury atoms were bound per protein molecule, probably with its imidazole groups. Another metal ion with which the reaction of the -SH group of biologlically important compounds finds some useful application for the estimation of SH groups is the silver[97-100].

The interaction of copper with serum albumin has been systematically investigated in Klotz Laboratories[101-103] The following informations were obtained on the basis of equilibrium dialysis studies[39-40]. (i) At copper concentration $1C^{-4}$ M and at pH 4.8, one copper was bound per

protein molecule, (ii) the number of copper atoms bound increased from 10-11, when the metal ion concentration was increased to 10^{-2}M; (iii) Spectrophotometric studies with simple ligands as well as methylated proteins[40] revealed[60] that copper combined with the carboxyl groups at pH 4.8 and with the imidazole groups at pH 6.5 (the spectra of the protein complex resembling that of copper imidazole complexes[104]). Studies carried out at higher pH 9.6, showed that copper combined with the amino groups and in strongly basic solution each copper was bound to four peptide nitrogen[105-106]. Qualitative information on the combination of copper with α-casein, β-lactoglobin, γ-globin and lysozyme was also obtained by Klotz and co-workers.[41-60]

Another class of metal protein complexes which have been studied in detail are those of zinc and cadmium, Gurd and Goodman[35-36] have shown that the zinc ion combined with the imidazole groups of the protein in the pH range 5 to 6.8 reversibly at zero degree but irreversibly at higher temperatures viz., 25° and 37°. Tanford[51] employing the polarographic technique, also showed that the imidazole groups were the principal sites for zinc and cadmium binding to bovine serum albumin. Moreover, Gurd[107] on the basis of equilibrium dialysis studies was able to show that cadmium was bound somewhat more strongly than zinc. The nature of another important zinc complex with protein, viz., insulin has been investigated by

Tanford and Epstein[46] by pH metric and polarographic methods. They concluded that each zinc was bound to two imidazole groups below pH 6.5. Lead ion behave differently than copper, zinc and cadmium towards albumin. Gurd and Murray[37] observed complete precipitation of lead-protein complex even at pH 3 and they further found that each lead ion was bound to a single carboxyl group with association constant 68-87, nearly the same as reported for the association of lead with acetic acid[108](100). These results indicated that as many as 45 lead ions were bound upto pH 5.1. Lal and Rao, employing electrometric[54,68] and equilibrium dialysis[69] methods have studied in detail the combination of copper zinc, cadmium, cobalt and nickel with bovine serum albumin and have concluded that cobalt and possibly nickel were bound to free carboxyl groups, whereas the first three metals were bound primarily to the imidazole site. That nickel definitely takes part in the reaction has recently been confirmed by Rao[109].

Amongst the less heavier metal ions the interaction of calcium with serum albumin has been extensively investigated. Weir and Hastings[25] pointed out that serum albumin combined with calcium with intrinsic association constant 2.11 at pH 7.5. Using perm selective membranes Carr[44] obtained a higher value, viz., 2.43 which was ascribed to Donnon effect by Katz and Klotz.[42] The effect of hydrogen ion concentration on the dissociation of the calcium albuminate has recently

been investigated by Peterson, Crismon and Feigen[110]. Other proteins whose reaction with calcium have been investigated are haemoglobin, egg albumin, and lysozyme (studied potentiometrically by Carr[44]). Recently the interaction of this metal with β-Lactoglobin has been reported by Zittle et al[111].

Attention to irreversible binding of some heavy metal ions with proteins have already been drawn. The interaction of iron with conalbumin[62,63,112] is expected to be irreversible due to the formation of polynuclear complexes. It has been found that the reaction can be made reversible by employing the citrate complex of the metal instead of the simple ion in the bicarbonate medium. Werner and Weber[63] proposed a mechanism for the reaction by suggesting that the citrate anion and the protein could very well exchange iron and copper in a reversible manner and that phenoxyl groups (from tyrosine residue) were predominently involved in chelation.

The investigations on metal protein interactions, surveyed so far, have given a quantitative aspect of the interactions of albumins. This aspect has, however, not been studied with other proteins, particularly gelatin which is both physiologically and industrially important compound. The history of the interaction of metal ions with gelatin can be traced as far back as 1918 when Loeb[113] pointed out that gelatin, above its isoelectric point combined with silver ions and that hydrogen ion

must be competed[114] by the metal ions in order that the latter should interact with gelatin. The conductometric and potentiometric measurements of Pauli and Matula[115] also gave indications of similar reactions of the metal ion with this protein. In view of the great importance of this reaction in Photography, it has been widely investigated in recent years[116-119] providing evidence that the metal ion coordinates with the nitrogen atom of the imidazole groups[119]. Other metal ions whose reaction with gelatin have been studied are lithium, potassium, zinc, Manganese, copper etc., employing equilibrium dialysis technique (Northrop and Kunitz[34]). Bonino and co-workers[120] studied the interaction of cobalt with gelatin by means of potentiometric methods and demonstrated the dependence of cobalt-gelatin reaction on pH, the combination being minimum at the isoelectric point. The possible combination of cobalt with gelatin was also shown by electrophoretic studies.[121]

The chromium gelatin reaction is another example of a sluggish reaction. The reaction between collagen and basic chromium finds most wide applications in chrome tanning of animal hide. Because of its great importance, the reaction has been thoroughly investigated by Gustavson[122-123] Pouradier[124] and Schulman[125]. Accumulative evidences regarding the chromium binding reveal that the protein offers carboxyl groups as the principal sites for coordination with metal ions. This view point was supported by (i) the close similarities between the spectra of

chromium-glycine and chromium-gelatin complexes and (ii) masking the carboxyl groups by methylation and noting the decrease in the uptake of metal ion by the protein. Evidence is also forthcoming for the binding of chromium with other groups on ageing the metal-protein mixture and some sort of chelate structure of high stability has been postulated. Besides chromium, the other metal which has been studied in detail is copper[34]. In this connection mention may be made of the work of Wolff and Falleb[126], who by spectrophotometric method have shown a close resemblance between the spectra of copper gelatin complex and copper-peptide complex and that the particular configuration of gelatin was responsible for its greater uptake of copper ion than the peptide having the same number of free terminal groups. A greater insight in the mode of binding is now available by considering the biuret reaction of gelatin[127-129]. It has been invariably pointed out that the peptide nitrogen and oxygen atoms are involved in such reactions.

From the foregoing review it appears, that very little attention has been paid for the quantitative elucidation of the binding of metal ions with proteins like gelatin, casein, haemoglobin etc. Extensive studies in this direction were, therefore, planned, for the first time in this laboratory by Malik and Co-workers[49,56,58,64-67,130] employing transfusion gelatin (a protein which is important from pharmacological[131] point of view and has a simpler

configuration and known molecular weight). Another new and interesting aspect investigated by these workers was to determine the availability of metal ions from their hydrous oxide sols for interaction with the proteins.[132-135] The work described in this thesis deals with the extensive studies and advancement made in this direction.

Statement of the Problem

The information contained in the following pages have been divided into four parts.

1. Part one deals with the quantitative studies on the binding of metal ions with transfusion gelatin employing polarographic, pH-metric and equilibrium dialysis techniques. The metal ions taken are Pb(ii), Sn(ii), Cu(ii), Cd(ii), Zn(ii, Co(ii) and Ni(ii).

2. Second part contains spectrophotometric investigations on the interaction of Cr(iii) with transfusion gelatin and bovine serum albumin, and the biuret reaction of transfusion gelatin with Cu(ii) and Ni(ii). Some preliminary studies have also been carried out to indicate the influence of a number of bivalent metal ions viz., Cu(ii), Zn(ii), Cd(ii), Co(ii) and Pb(ii) on the absorption peak of Ni-transfusion gelatin complex at pH 12.0.

3. The results on the investigation of colloid-chemical behaviour during the mutual interaction of casein

and egg albumin with a number of metal ions (Al, Fe, Cr, Co, and Ni) and their hydrous oxide sols are presented in the third part. Here it is intended to explore the possibility of metal (both from the sol and electrolyte) entering into combination with the available sites on the protein molecule. pH-metry and viscosity methods are employed.

4. Last part of the thesis deals with the studies on the interaction of metals (Pb and Cr) with amino acids and peptides. Bjerrum's method is employed for the evaluation of stepwise as well as overall stability constants. The following amino acids are used.

L-proline, Glycine, L-Hydroxy proline, L-Asparagine, DL. Lysine, DL. Valine, threonine DL.:henylalanine, DL. Serine, DL. Methionine and Glycylglycine.

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P A R T I.

Quantitative studies on the interaction of metals with transfusion gelatin

(a) CHAPTER I.

Polarographic studies on the interaction of lead and tin with transfusion gelatin.

I N T R O D U C T I O N

Of the various electrometric techniques employed in investigating physico-chemical phenomena, the polarographic method[1] occupies a unique position as far as its universal applicability in different branches of Chemistry is concerned. It has not only helped in solving the various intricacies of inorganic reaction, but is also being successfully used in reactions involving organic compounds, and products of biological importance. Recent literature abounds in references on amino acids, polypeptides, native and denatured proteins (e.g., vide Brdicka reaction[2-6]) which provide enough evidence for the accuracy of the polarographic estimations, the method even claims its usefulness in the diagonsis of diseases, especially in cancer.[7]

Apart from the practical utility of the polarographic method, one finds that it also provides a fruitful means of studying problems of fundamental interest, in the fields of electrochemistry and chemical kinetics (e.g., phenomena of over voltage, electrocapillary force acting at the mercury-electrolyte interface, kinetics of electrode reactions[8-13] etc.). The role of polarograp

in co-ordination Chemistry is by no means less impressive. On the other hand, recent work[14-17] on the metal complexes of simple organic substances, amino acids and peptides has established the superiority of the method over other prevalent methods. In this connection the work of Heyrovsky and Ilkovic[18] on the reduction of complex metal ions at dropping mercury electrode and that of Lingane[19] and Lingane and Kolthoff[2] deserve special merit. Their interpretation based on the shift in half wave potential, however, does not apply when dealing with more complex systems like the metal-protein complexes, which form the main theme of the work presented here.

The proteins exert a two-fold influence on the reduction waves of metal ions, namely the suppression of the polarographic maxima and marked reduction in the diffusion current. The latter effect has been ascribed to a number of factors, viz., adsorption of proteins on mercury drops[21-23], increase in viscosity of the medium[24] and complex formation between metal and protein. According to Zuman[21] and also Tanford[22], the decrease in diffusion current is primarily due to complex formation, provided the investigations are carried out in the higher pH range where the possibility of adsorption of the negatively charged protein molecule

on mercury drop is very remote. Scatchard[25] gave the following relationship for metal-protein interaction:

$$Ke^{-2ZM^{Z_P}W} = \frac{\bar{V}M}{(n - \bar{V}M - \bar{V}H)C}$$

where VM and VH are the number of metal and hydrogen ions bound per protein molecule, n is the total number of reactive sites, C_F is the free metal concentration and K is the intrinsic association constant. If the viscosity factor is not involved, VM when computed polarographically (considering the depression of diffusion current) would be independent of protein concentration, a test which has proved to be valid in a number of cases.

On the basis of the above reasoning Tanford[22,26], for the first time successfully applied the polarographi method to investigate the nature of the metal-protein interaction. The salient features of these investigation are: (i) for a given metal and protein concentration, the ratio $(id)/(id)_o$, where (id) and $(id)_o$ are the diffusion current of metal ion in presence and absence of protein, should decrease with increase in pH and attain a limiting value; (ii) for a given metal concentration the ratio $(id)/(id)_o$ should decrease with increasing amount of protein and attain a limiting

value not zero[28], and (iii) the ratio should decrease with decreasing metal ion. The validity of Tanford's method (which mainly deals with the interaction of heavy metal ions with serum albumin and insulin) is forthcoming from the work of Rao and Lal[29] (Zinc and Cadmium complexes of bovine serum albumin in buffer media) and Saroff and Mark[30] (Zinc and mercury-serum albumin complexes. The complex formation was accompanied by a detectable shift in half wave potential and the binding data calculated from diffusion current measurements were agreed well with equilibrium dialysis studies).

From what has been cited above it is evident that the polarographic method has not been fully exploited for the study of metal complexes of fibrous proteins. However, more than a decade ago Tayler and Smith[31] made an interesting observation that the diffusion current of lead ion was markedly decreased in presence of increasing amount of gelatin. An indirect approach towards the lead complexes of egg albumin, haemoglobin and gelatin has been made by Suzutani[32], who estimated the free lead polarographically after the complete adsorption of these complexes on animal charcoal. Tachibana et al[33] estimated the free lead ion in presence of gelatin. Recently the successful

application of polarographic technique to the transfusion gelatin complexes of copper, cadmium and zinc,[34-35] coined with fact that, Tayler and Smith's observations remained unnoticed and no attempt has been made so far, to investigate it quantitatively in terms of lead-gelatin combination, stimulated to carry out the present studies. The protein chosen for this purpose was transfusion gelatin[36] in view of its better characterisation in terms of its hydrogen ion equilibria and molecular weight. The technique employed was the same as reported earlier from these laboratories.

Tin(ii) plays an important part in biochemical reactions connected with animal metabolism and enzyme activity. Their role has not been fully understood. It was, therefore, thought worthwhile to work it up polarographically using transfusion gelatin. The results on the binding data revealed that like lead tin combines primarily with the carboxyl groups of transfusion gelatin.

E X P E R I M E N T A L

Apparatus:

The polarograph used, was a Fisher Elecdropode (Fisher Scientific Co. Pittsburgh. Pa) in conjunction with the Multiflex Galvanometer (Type MGF2) in the external circuit. An H-shaped polarographic cell was designed as recommended by Tanford to hold 2 to 3 m.l. of solution. It was found to be suitable for deareation of protein solution without denaturation and subsequent measurement at D.M.E. An inert atmosphere was ensured by passing pure nitrogen (through alkaline pyrogallol and chromous chloride) for about 15 to 20 mits. for each run. Triply distilled mercury (A.R.) was used for dropping electrode. Diffusion current was measured for each increment of potential keeping the cell in a constant temperature bath (Townson and Mercer Co.Croydon) at $t \pm 0.1^{o}C$. The capillary used, had a flow rate of approximately 2.2 mg/sec with a drop time of 3.6 to 3.8 sec. The rate of flow of mercury drops was determined by Lingane method[37] using a stop watch reading one tenth of a second. Since the capillary characteristics (m,t) have a pronounced effect on diffusion current (which is directly proportional to $m^{2/3} t^{1/6}$) according to Ilkovic equation, these factors were, therefore, controlled

carefully throughout these investigations.

The pH of the buffers and mixtures of metal and protein solution were measured on a Backman Model G pH-meter using general purpose glass electrode.

Solutions and Reagents

Transfusion gelatin (concentration 6 %, Molecular weight 75,000) supplied by the Director, National Chemical Laboratories, Poona, India, was used throughout these investigations.

Chemically pure samples of metal salts (E Merdk) were dissolved without further purification, in doubly distilled water (distilled in all glass apparatus) to get their respective solutions. The lead content was determined by titrating it against EDTA using E Black T indicator[38], and tin content was estimated volumetrically against potassium iodate using carbontetra chloride indicator[39].

Walpole Acetate[40] and ammonium acetate-ammonia buffers were prepared by mixing 0.2M in each sodium acetate and acetic acid, and 0.2M in each ammonium acetate and ammonia respectively. The ammonium acetate

ammonia buffer used to maintain the pH of stannous-transfusion gelatin mixtures was prepared from 2.0M in each ammonium acetate and acetic acid.

A.R. samples of potassium nitrate and potassium chloride were employed to prepare 1.5M solutions. These solutions were used to maintain constant ionic strength.

Procedure

The following sets were subjected to polarographic analysis.

A. (i) 1 m.l. of lead nitrate (0.01M) solution, 8 m.l. of acetate buffers of pH 3.72, 4.45, 4.8, 5.2 and 5.57 and 2 m.l. of protein solution were mixed and the total volume made upto 20 m.l. by adding potassium nitrate and distilled water, so that the ionic strength was adjusted to 0.15 in each case. Similar sets were analysed, in which the protein was replaced by 2 m.l. of water. Thus the diffusion current of lead ion at different pH values in absence and presence of protein were measured At higher pH-values, viz., 5.5, 5.95, 6.35 and 6.8 ammonium acetate ammonia buffer was employed.

(ii) Varying amounts of protein (concentration given

in tables), were mixed at a given concentration of lead nitrate (1 m.l. of 0.01M) and acetate buffer (8 m.l. of pH 5.57) was added and the total volume made upto 20 m.l. with the ionic strength being kept at 0.15 in each case. Solution of high protein content ($1.8 - 2.4 \times 10^{-8}$M) precipitated within an hour. In such cases analysis was carried out immediately after mixing.

(iii) Varying amounts of lead nitrate (concentration given in tables), were mixed with a fixed amount of transfusion gelatin, and acetate buffer (8 m.l. of pH 5.57) was added keeping the total volume 20 m.l. and ionic strength at 0.15. Similar sets were analysed in which the protein was replaced by distilled water. Thus the diffusion current of metal ions in presence and absence of protein was recorded in each case.

B. (i) At a given metal (stannous chloride) and protein concentration, ammonium acetate buffers of pH-values, viz., 3.5, 3.9, 4.3, 4.6, 5.0 and 5.3 were mixed and total volume made upto 20 m.l. by adding water and requisite amount of potassium chloride, thus maintaining the ionic strength at 0.15. Similar set of solution in which protein was replaced by distilled water was also analysed to obtained the diffusion current in absence of protein.

(ii) Varying concentration of protein (given in the tables) were mixed at a given concentration of stannous chloride and buffer of pH 5.0 was added. The total volume made upto 20 m.l. keeping the ionic strength at 0.15.

(iii) Varying amounts of stannous chloride (given in tables) were mixed with a fixed amount of transfusion gelatin, 8 m.l. buffer of pH 5.0 was added and total volume made upto 20 m.l. with the ionic strength being kept at 0.15 in each case. Similar set in which the protein was replaced by distilled water was also analysed. Thus the diffusion current of metal ions in presence and absence of protein was measured.

The reversibility of the waves obtained, were tested by Tomes equation.[41] In case of stannous chloride only the Cathodic waves with a half wave potential - 0.63 volts vs S.C.E.[42] were considered.

TABLE 1.

Concentration of lead nitrate = 0.5×10^{-3} M

Concentration of transfusion gelatin = 0.6 % (0.8×10^{-4} M)

Total volume = 20 m.l. Total ionic strength = 0.15

Acetate buffer Temperature 30 ± 0.1°C

Applied Potential (Volts)	Current 4×10^{-8} Amperes			
	$(id)_o$	pH 3.72 (id)	pH 4.45 (id)	pH 4.8 (id)
0	- 1.6	- 2.6	- 3.2	- 3.2
0.10	- 0.5	- 2.0	- 2.5	- 2.0
0.20	- 1.0	- 1.5	- 2.0	- 1.8
0.30	- 1.5	- 1.0	- 1.4	- 1.5
0.35	4.6	- 1.8	- 1.0	- 0.6
0.38	16.5	12.6	11.2	10.6
0.40	27.5	21.8	21.2	20.6
0.45	36.5	30.2	30.0	30.5
0.50	37.0	31.2	30.8	31.2
0.55	37.2	31.5	31.2	31.4
0.60	37.5	31.6	31.4	31.5
0.70	38.0	31.8	31.5	31.5
0.80	38.2	32.0	31.6	31.6
0.90	38.5	32.4	31.8	31.8

(Fig. 1)

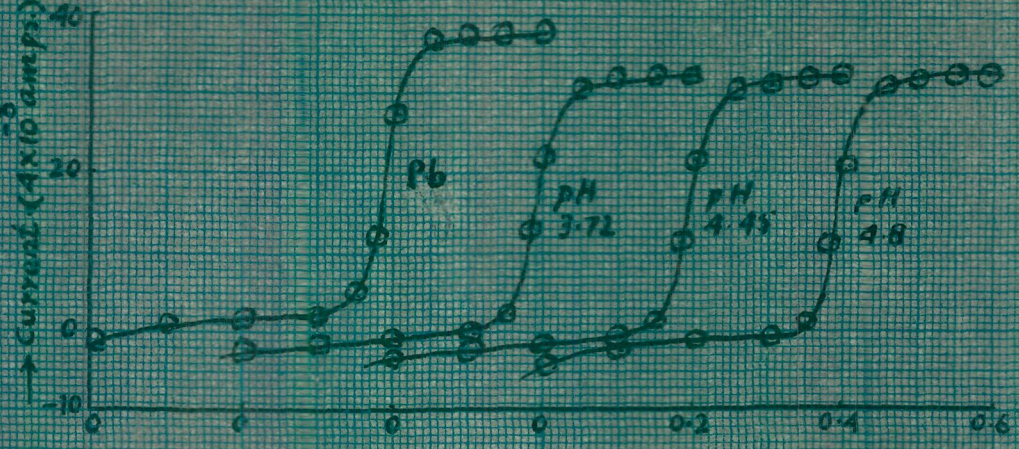


Fig 1 —> Pot. applied in Volts

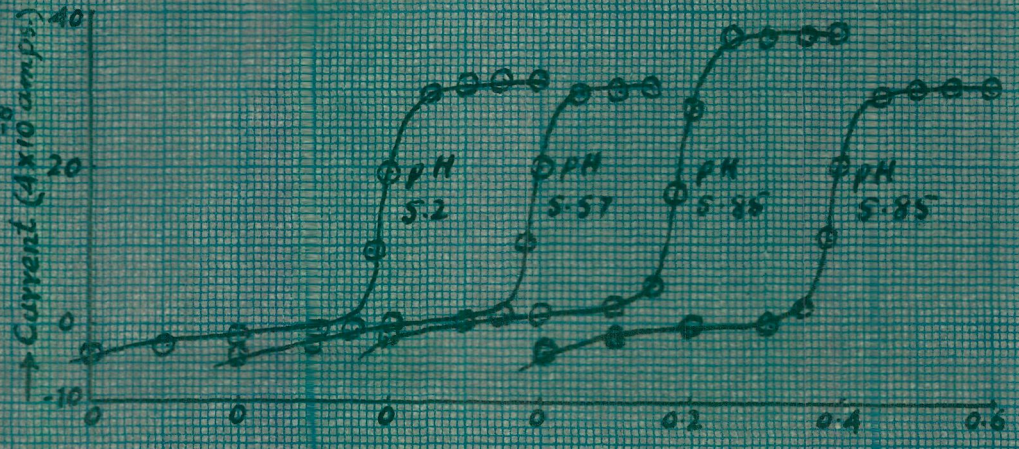


Fig 2 —> Pot. applied in Volts

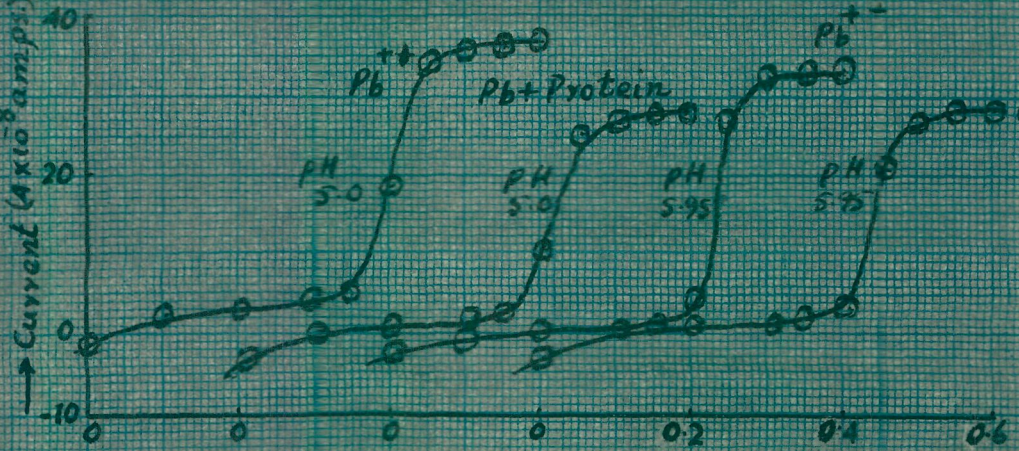


Fig 3 —> Pot. applied in Volts

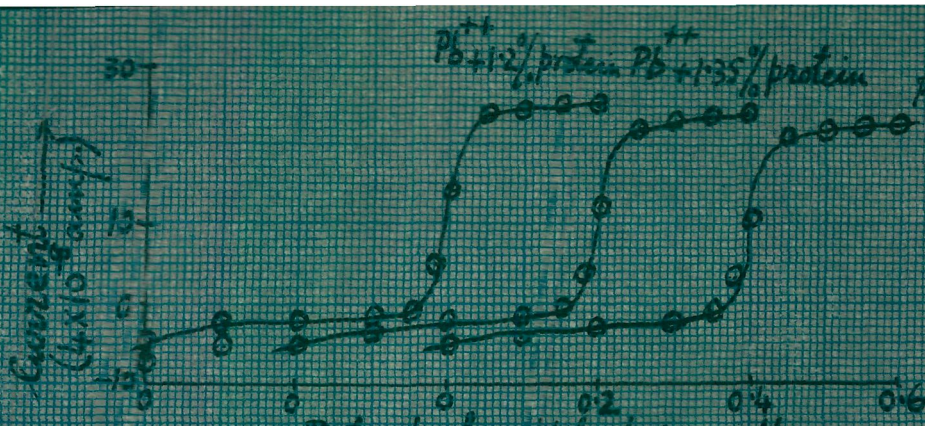


Fig 7

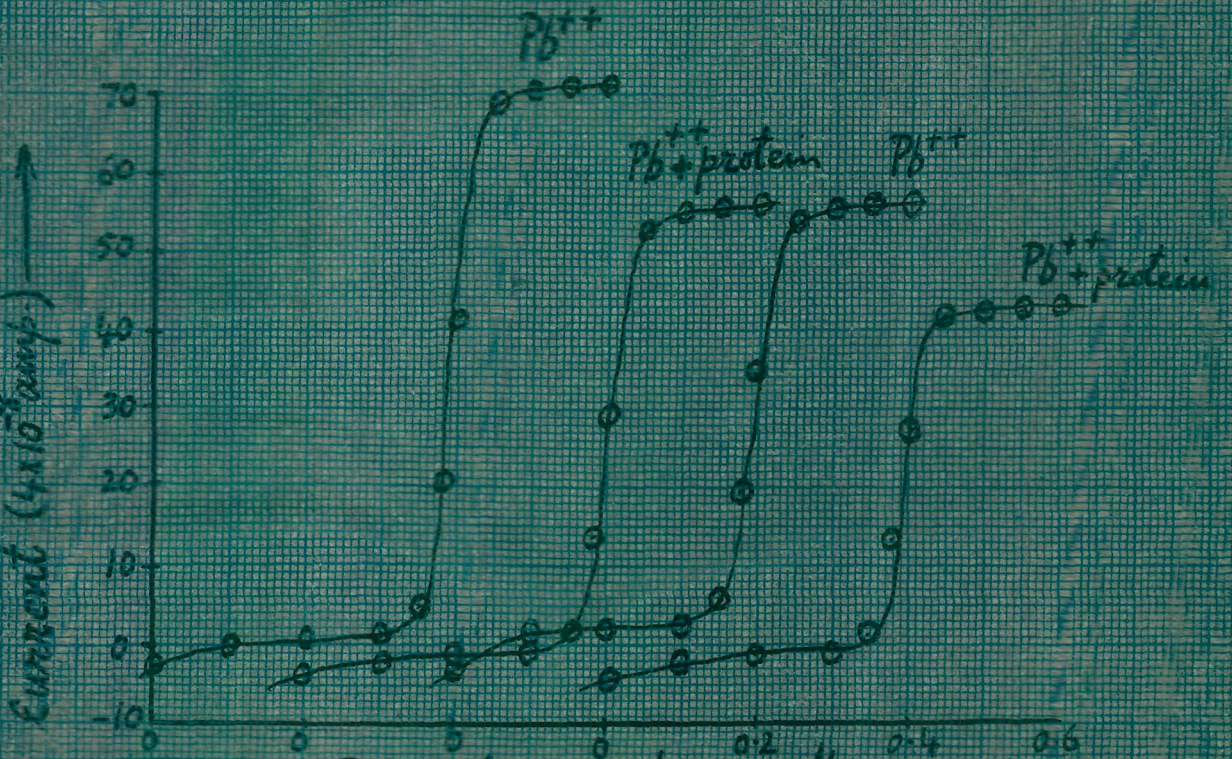


Fig 8

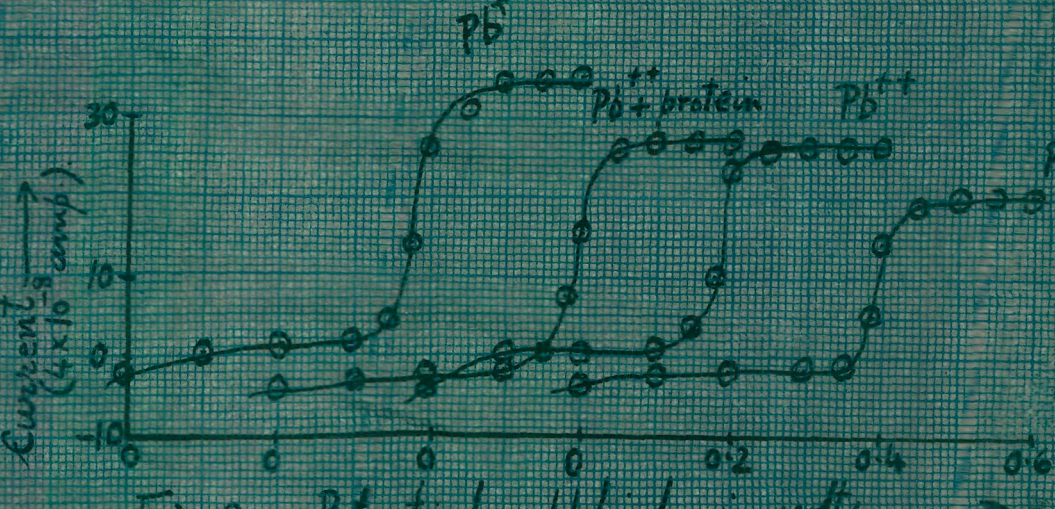


Fig 9

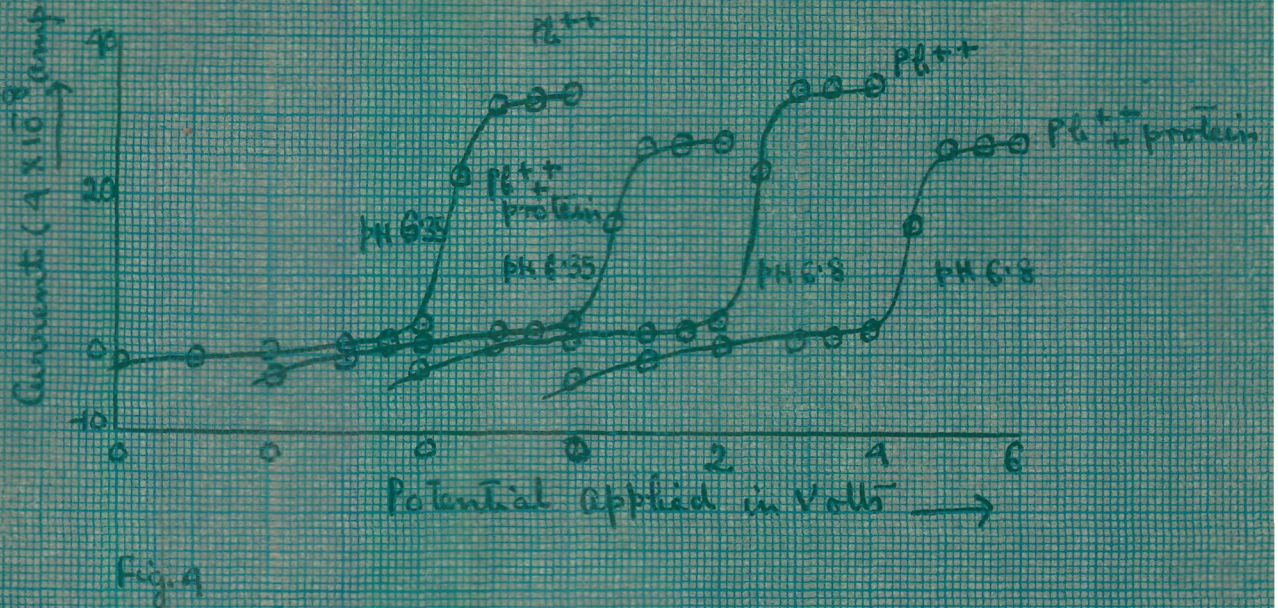


Fig. 4

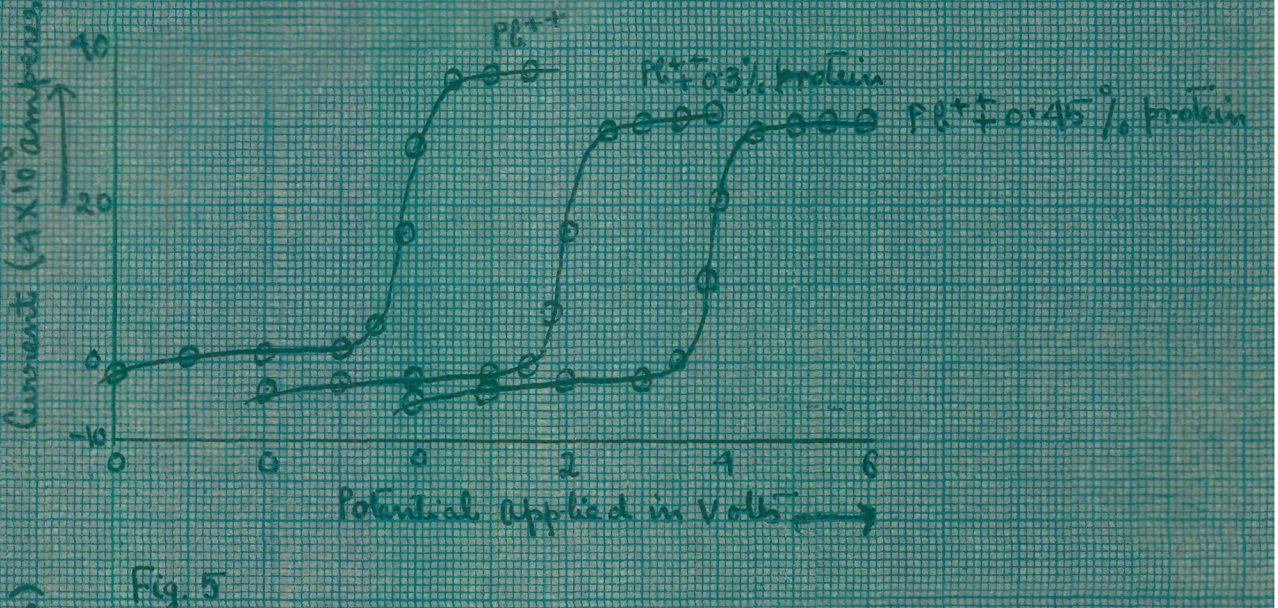


Fig. 5

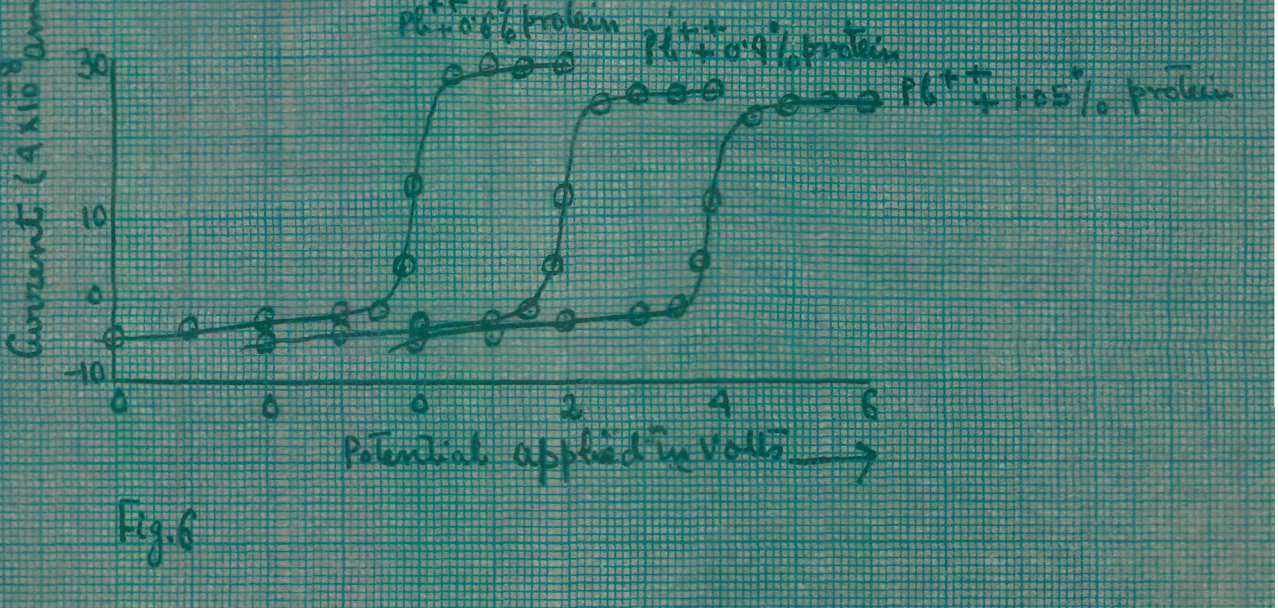
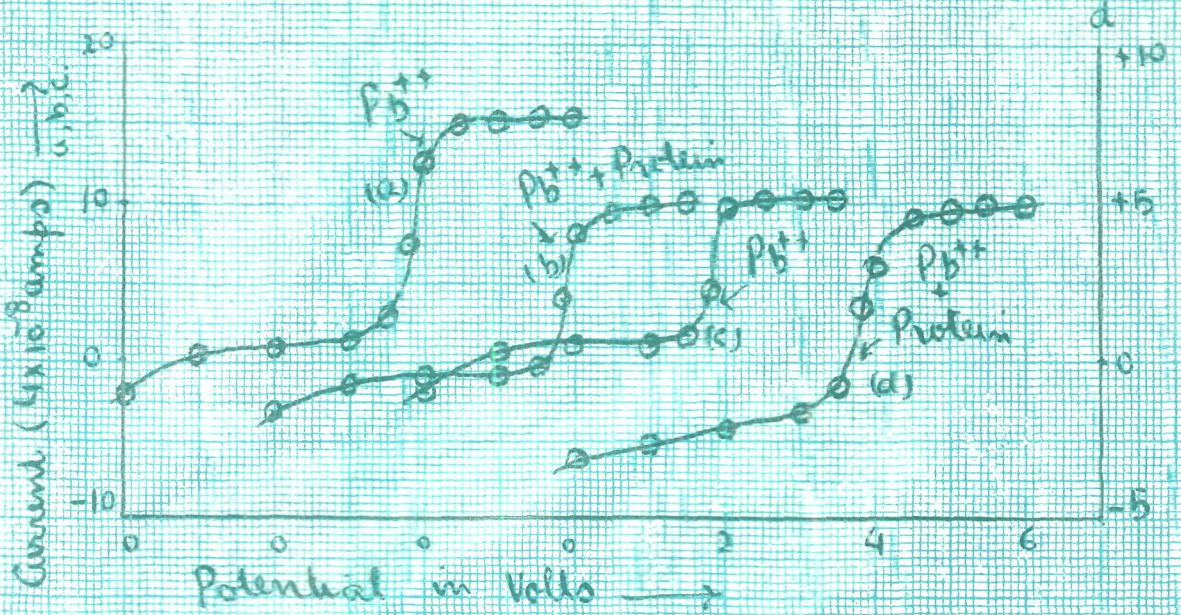
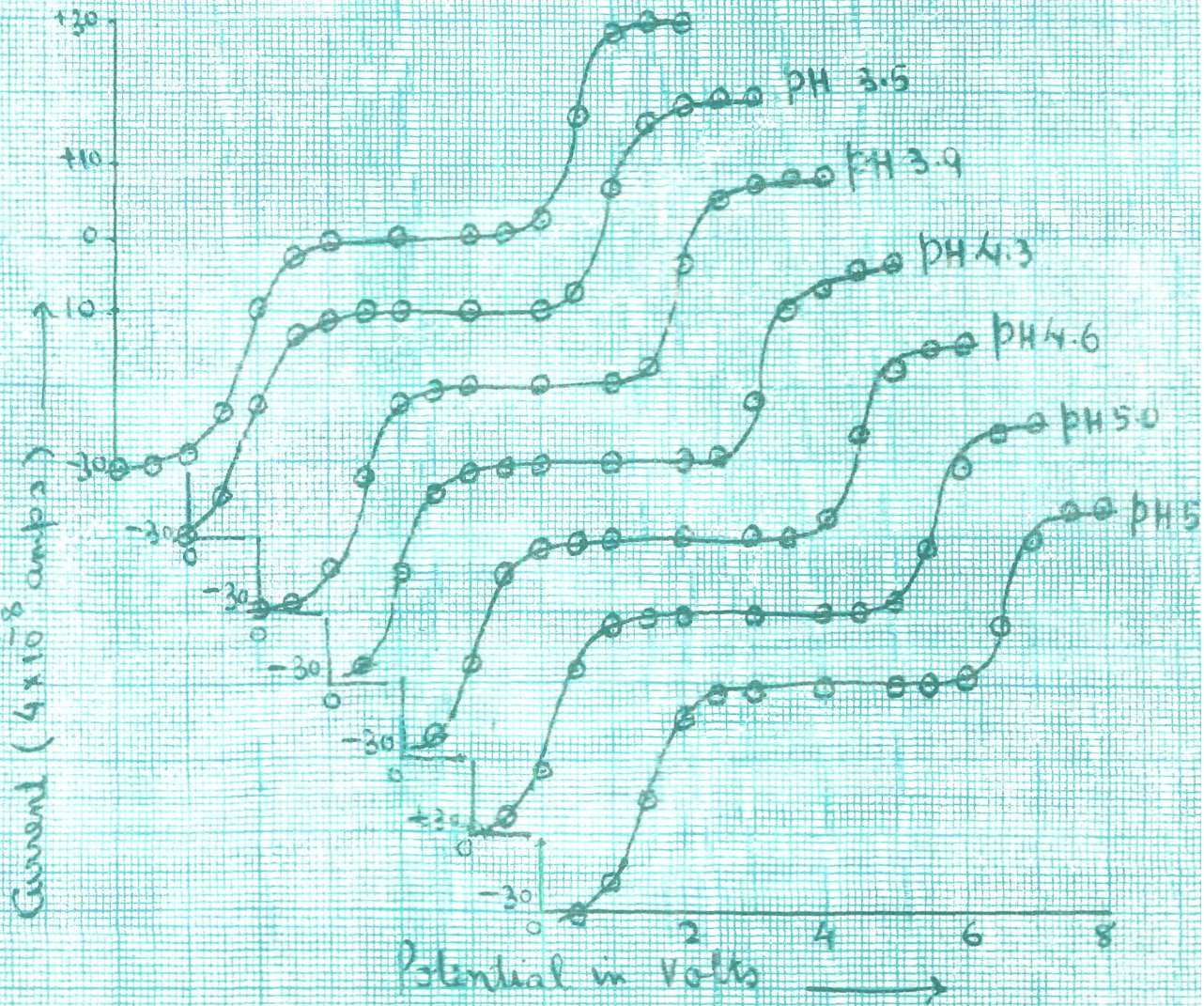


Fig. 6


Current (4×10^{-8} amps) →
+20
+10
0
-10
d
+10
+5
0
-5
Pb^{++}
(a)
Pb^{++} + Protein
(b)
Pb^{++}
(c)
Pb^{++} + Protein
(d)
0
2
4
6
Potential in Volts →


Current (4×10^{-8} amps) →
+30
+10
0
-10
-30
pH 3.6
pH 3.9
pH 4.3
pH 4.6
pH 5.0
pH 5
2
4
6
8
Potential in Volts →

Figs. 11, 12

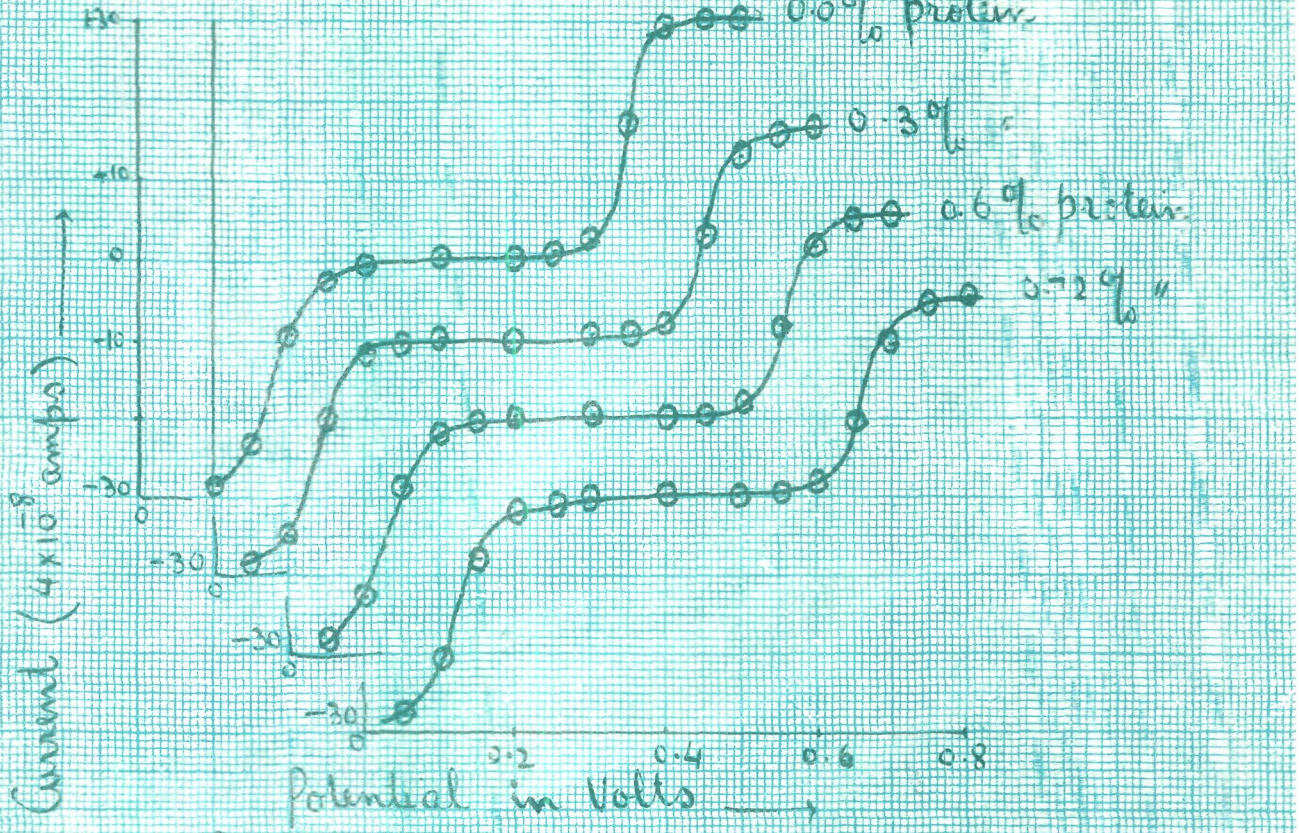
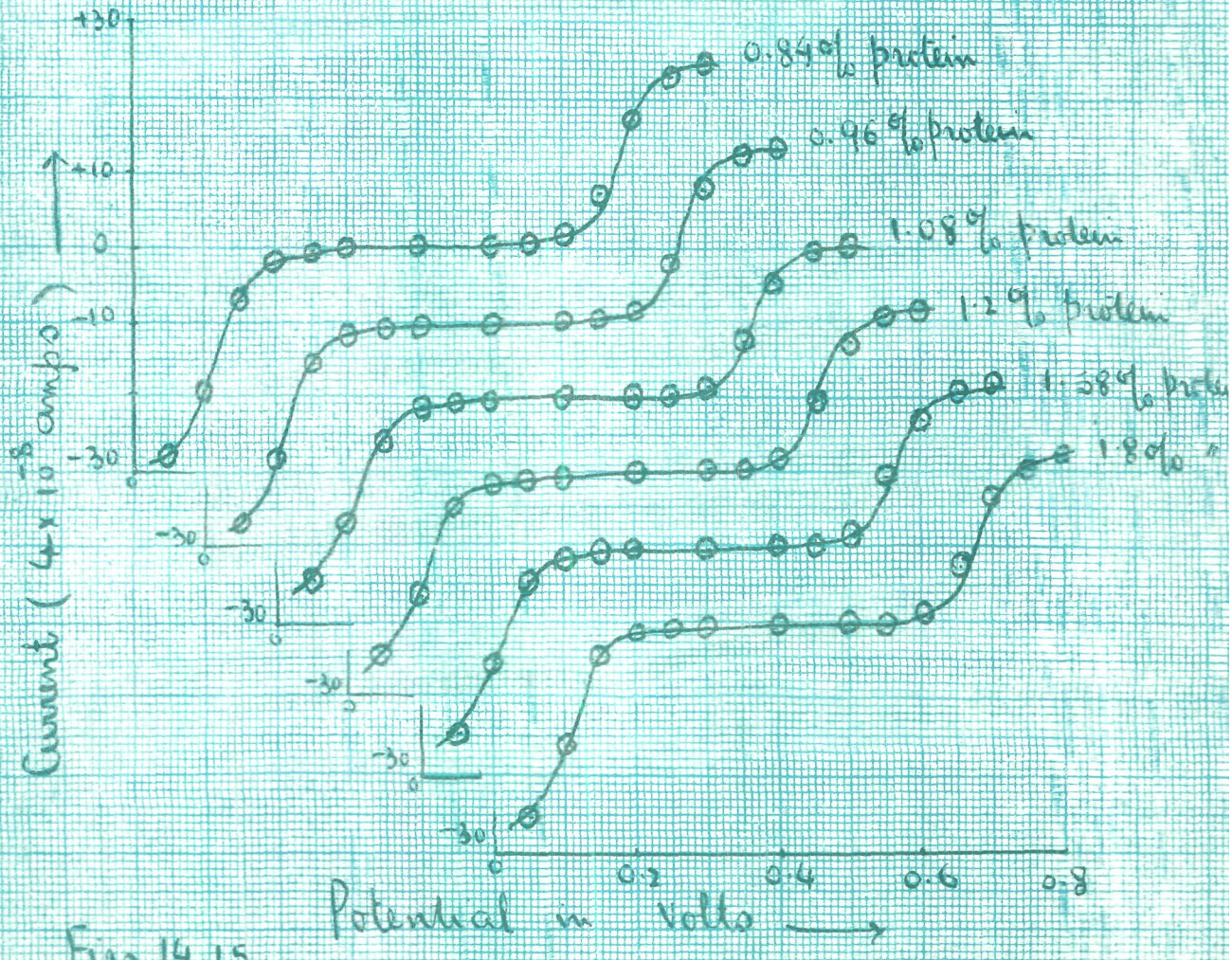


Fig. 13



Figs 14, 15.

T A B L E 2.

Concentration of lead nitrate = 0.5×10^{-3}M

Concentration of transfusion gelatin = 0.6 % (0.8×10^{-4}M)

Total volume = 20 m.l. Total ionic strength = 0.15

Acetate buffer Temperature 30 ± 0.1°C

Applied Potential (Volts)	Current 4×10^{-8} Amperes			
	pH 5.2 (id)	pH 5.57 (id)	pH 5.85 $(id)_o$	pH 5.85 (id)
0.0	- 3.4	- 3.2	- 1.5	- 3.4
0.10	- 2.6	- 2.0	0	- 2.0
0.20	- 2.2	- 0.8	0.8	- 0.8
0.30	- 1.8	- 0.5	1.4	- 0.5
0.35	- 0.4	1.4	4.6	1.4
0.38	9.0	10.0	16.2	10.0
0.40	19.0	19.5	27.0	19.4
0.45	29.4	29.0	36.0	28.8
0.50	30.0	29.6	36.4	29.0
0.55	30.4	29.8	36.5	29.5
0.60	30.4	30.0	36.6	29.8
0.70	30.5	30.2	36.8	30.0
0.80	30.6	30.5	37.0	30.4
0.90	30.6	30.6	37.2	30.5

(Fig. 2)

TABLE 3.

Concentration of lead nitrate = 0.4×10^{-3}M

Concentration of transfusion gelatin = 0.6 %(0.8×10^{-4}M)

Total volume = 20 m.l. Total ionic strength 0.15

Ammonium acetate - acetic buffer Temperature = 30 ± 0.1°C

Applied Potential (Volts)	Current (4×10^{-8} Amperes)			
	pH 5.0		pH 5.95	
	$(id)_o$	(id)	$(id)_o$	(id)
0.0	- 0.5	- 2.5	- 1.8	- 2.8
0.10	3.0	0.5	- 0.5	0
0.20	3.5	1.0	0.5	1.5
0.30	4.2	2.0	0.8	1.6
0.35	5.0	2.5	1.0	1.8
0.40	18.8	10.0	4.0	3.0
0.45	34.8	25.0	27.0	20.0
0.50	36.2	27.5	32.2	26.5
0.55	36.5	28.0	32.8	27.5
0.60	37.0	28.5	33.0	27.6
0.65	37.4	28.6	33.4	27.8
0.70	37.5	28.6	33.5	28.0
0.80	38.0	28.6	34.0	28.4
0.90	38.0	29.0	34.5	29.0

(Fig. 3)

TABLE 4.

Concentration of lead nitrate = 0.4×10^{-3}M

Concentration of transfusion gelatin = 0.6 % (0.8×10^{-4}M)

Total volume = 20 m.l. Total ionic strength = 0.15

Ammonium acetate-acetic acid buffer Temperature 30 ± 0.1°C

Applied Potential (Volts)	Current (4×10^{-8} Amperes)			
	pH 6.35		pH 6.8	
	$(id)_o$	(id)	$(id)_o$	(id)
0.0	- 1.8	- 2.5	- 1.4	- 3.0
0.10	- 0.8	0	1.0	- 0.5
0.20	0.4	2.0	2.0	1.8
0.30	0.8	2.4	2.2	2.4
0.35	1.0	2.5	2.5	2.5
0.40	3.2	3.0	4.4	3.0
0.45	22.0	16.5	22.5	16.5
0.50	31.5	26.0	32.8	26.0
0.55	32.0	26.8	33.5	26.5
0.60	32.2	27.0	33.8	26.8
0.65	32.5	27.2	34.0	27.0
0.70	32.8	27.4	34.2	27.2
0.80	33.4	27.5	35.0	27.2
0.90	34.0	27.5	35.2	27.4

(Fig. 4)

TABLE 5.

Concentration of lead nitrate = 0.5×10^{-3}M

pH of acetate buffer = 5.57

Total volume = 20 m.l. Total ionic strength = 0.15

Temperature 30 ± 0.1°C

Applied Potential (Volts)	Current (4×10^{-8} Amperes)		
	0.0% protein $(id)_o$	0.30% protein (id)	0.45% protein (id)
0.0	- 1.6	- 3.8	- 4.2
0.10	0.5	- 3.2	- 3.5
0.20	1.0	- 2.5	- 2.8
0.30	1.5	- 2.0	- 2.5
0.35	4.6	- 0.8	0
0.38	16.5	6.4	10.2
0.40	27.5	16.5	20.5
0.45	36.5	29.4	29.5
0.50	37.0	30.5	30.5
0.55	37.2	31.0	30.8
0.60	37.5	31.5	31.0
0.70	38.0	31.5	31.5
0.80	38.2	31.8	31.5
0.90	38.5	32.0	31.5

(Fig. 5)

TABLE 6.

Concentration of lead nitrate = 0.5×10^{-3}M

pH of acetate buffer = 5.57

Total volume = 20 m.l. Total ionic strength = 0.15

Temperature - 30 ± 0.1°C

Applied Potential (Volts)	Current (4×10^{-8} Amperes)		
	0.60% Protein (id)	0.90% Protein (id)	1.05% Protein (id)
0.0	- 4.5	- 4.5	- 4.5
0.10	- 3.5	- 3.8	- 3.5
0.20	- 2.6	- 3.1	- 2.4
0.30	- 2.2	- 2.6	- 1.8
0.35	- 1.0	- 1.5	- 1.0
0.38	4.8	4.0	4.0
0.40	14.0	12.5	11.5
0.45	27.8	24.5	23.0
0.50	29.0	25.5	24.5
0.55	29.5	25.8	25.0
0.60	29.8	26.0	25.5
0.70	30.0	26.5	26.0
0.80	30.0	27.0	26.0
0.90	30.0	27.0	26.0

(Fig. 6)

TABLE 7.

Concentration of lead nitrate = 0.5×10^{-3}M

pH of acetate buffer = 5.57

Total volume = 20 m.l. Total ionic strength = 0.15

Temperature 30 ± 0.1°C

Applied Potential (Volts)	Current (4×10^{-8} Amperes)		
	1.20% Protein (id)	1.35% Protein (id)	1.5% Protein (id)
0.0	- 4.6	- 4.8	- 5.0
0.10	- 3.6	- 3.5	- 4.0
0.20	- 2.8	- 2.5	- 3.0
0.30	- 2.2	- 2.0	- 2.4
0.35	- 1.2	- 1.0	- 1.5
0.38	4.8	3.8	2.8
0.40	13.4	11.4	9.8
0.45	23.5	21.8	20.5
0.50	24.0	23.0	21.5
0.55	24.5	23.5	22.0
0.60	24.8	23.8	22.5
0.70	25.0	24.0	23.0
0.80	25.5	24.5	23.5
0.90	25.5	24.8	23.8

(Fig. 7.)

TABLE 8.

Concentration of transfusion gelatin = 0.60%(0.8×10^{-4}M)

pH of acetate buffer = 5.57

Total volume = 20 m.l. Total ionic strength = 0.15

Temperature 30 ± 0.1°C

Applied Potential (Volts)	Current (4×10^{-8} Amperes)			
	1.0×10^{-3}M Pb^{--}		0.75×10^{-3}M Pb^{--}	
	$(id)_o$	(id)	$(id)_o$	(id)
0.0	- 2.4	- 3.2	- 1.8	- 3.5
0.10	- 0.5	- 2.4	1.0	- 2.5
0.20	0.5	- 1.5	2.2	- 1.6
0.30	1.0	- 1.0	2.5	- 1.0
0.35	4.2	1.5	6.0	1.4
0.38	20.6	13.4	19.4	13.4
0.40	41.0	29.0	34.6	27.0
0.45	68.5	52.5	53.8	41.5
0.50	70.5	55.0	55.4	42.5
0.55	70.5	55.5	55.5	42.6
0.60	70.8	55.6	55.8	42.8
0.70	71.2	55.8	56.5	43.0
0.80	71.5	56.0	56.6	43.0
0.90	72.0	56.0	56.8	43.2

(Fig. 8.)

TABLE 9.

Concentration of transfusion gelatin = 0.6% (0.8×10^{-4}M)

pH of acetate buffer = 5.57

Total volume 30 m.l. Total ionic strength = 0.15

Temperature 30 ± 0.1°C

Applied Potential (Volts)	Current (4×10^{-8} Amperes)			
	0.5×10^{-3}M Pb^{--}		0.35×10^{-3}M Pb^{--}	
	$(id)_o$	(id)	$(id)_o$	(id)
0.0	- 2.0	- 3.4	- 2.5	- 3.0
0.10	0.8	- 2.5	0.8	- 2.0
0.20	1.6	- 1.8	1.0	- 1.5
0.30	2.0	- 1.5	1.5	- 1.0
0.35	4.5	0.4	4.0	- 0.5
0.38	14.5	7.4	10.5	5.2
0.40	26.2	16.0	23.5	14.2
0.45	31.5	26.5	26.0	19.2
0.50	35.0	27.4	26.5	20.0
0.55	35.4	27.5	26.8	20.4
0.60	35.5	27.6	27.0	20.5
0.70	35.6	27.8	27.2	20.6
0.80	35.8	28.0	27.2	20.8
0.90	35.8	28.0	27.2	20.8

(Fig. 9)

TABLE 10.

Concentration of transfusion gelatin = 0.6% (0.8×10^{-4}M)

pH of acetate buffer = 5.57

Total volume = 20 m.l. Total ionic strength = 0.15

Temperature 30 ± 0.1°C

Applied Potential (Volts)	Current (4×10^{-8} Amperes)			
	0.2×10^{-3}M Pb^{--}		0.1×10^{-3}M Pb^{--}	
	$(id)_o$	(id)	$(id)_o$	(id)
0.0	- 2.0	- 3.0	- 2.0	- 3.2
0.10	0.8	- 1.5	1.4	- 2.5
0.20	1.0	- 1.0	2.4	- 2.0
0.30	1.5	- 0.5	2.5	- 1.5
0.35	3.0	0	3.0	- 0.8
0.38	7.5	4.0	4.5	1.8
0.40	12.5	8.0	9.2	3.0
0.45	15.0	9.5	10.2	4.5
0.50	15.2	10.0	10.5	4.8
0.55	15.4	10.2	10.6	5.0
0.60	15.5	10.4	11.0	5.0
0.70	15.8	10.5	11.2	5.2
0.80	16.0	10.5	11.2	5.4
0.90	16.0	10.5	11.2	5.5

(Fig. 10)

TABLE 11.

Concentration of stannous chloride = 13.53×10^{-4} M

Concentration of transfusion gelatin = 0.72% (0.96×10^{-4} M)

Total volume = 20 m.l. Total ionic strength = 0.15

Ammonium acetate - acetatic acid buffer,
Temperature ± 30 0.1°C

Applied Potential (Volts)	Current (4×10^{-8} Amperes)			
	$(id)_o$	pH 3.5 (id)	pH 3.9 (id)	pH 4.3 (id)
0.0	- 31.0	- 30.0	- 29.5	- 29.5
0.05	- 30.5	- 24.5	- 29.0	- 27.0
0.10	- 29.5	- 12.5	- 24.0	- 14.5
0.15	- 23.5	- 2.8	- 12.0	- 4.0
0.20	- 10.0	- 0.5	- 2.5	- 0.8
0.25	- 2.5	0	- 0.4	- 0.2
0.30	- 0.5	0.4	0	0
0.40	0	0.5	0.6	0.4
0.50	0.4	0.5	0.8	0.5
0.55	0.6	3.0	2.8	1.0
0.60	2.5	17.0	16.5	8.5
0.65	17.0	25.8	25.2	20.5
0.70	29.0	28.0	27.5	23.5
0.75	30.0	29.0	28.5	26.5
0.80	30.5	29.4	29.0	27.8
0.90	31.0	29.8	29.2	28.0
1.00	32.0	30.0	29.8	28.5

(Fig. 11)

TABLE 12.

Concentration of stannous Chloride = 13.53×10^{-4}M

Concentration of transfusion gelatin = 0.72% (0.96×10^{-4}M)

Total volume = 20 m.l. Total ionic strength = 0.15

Ammonium acetate-acetatic acid buffer, Temperature $30 \pm 0.1^{\circ}$C

Applied Potential (Volts)	Current (4×10^{-8} Amperes)		
	pH 4.6 (id)	pH 5.0 (id)	pH 5.3 (id)
0.0	- 28.5	- 29.0	- 31.0
0.05	- 26.5	- 28.0	- 30.0
0.10	- 17.0	- 21.0	- 26.0
0.15	- 4.5	- 7.8	- 15.5
0.20	- 1.0	- 1.0	- 4.0
0.25	- 0.2	- 0.2	- 1.0
0.30	0	0	- 0.4
0.40	0.2	0.2	0
0.50	0.5	0.5	0.2
0.55	0.6	0.5	0.4
0.60	3.0	1.8	1.5
0.65	14.0	9.0	8.0
0.70	22.5	19.0	19.5
0.75	25.5	24.0	24.0
0.80	26.5	26.0	26.0
0.90	27.0	27.0	27.5
1.00	27.5	27.5	28.0

(Fig. 12)

TABLE 13.

Concentration of stannous Chloride = 13.53×10^{-4} M

pH of Ammonium acetate-acetic acid buffer = 5.0

Total volume = 20 m.l. Total ionic strength = 0.15

Temperature 30 ± 0.1°C

Applied Potential (Volts)	Current (4×10^{-8} Amperes)			
	0 % Protein	0.3 % Protein	0.6 % Protein	0.72 % Protein
0.0	- 31.0	- 30.0	- 29.5	- 29.0
0.05	- 30.5	- 29.0	- 28.8	- 28.0
0.10	- 29.0	- 25.0	- 22.5	- 21.0
0.15	- 23.5	- 10.4	- 9.5	- 7.8
0.20	- 10.0	- 2.0	- 2.0	- 1.5
0.25	- 2.5	- 0.4	- 0.4	- 0.2
0.30	- 0.5	0	0	0
0.40	0	0.2	0.4	0.2
0.50	0.4	0.5	0.5	0.5
0.55	0.6	0.5	0.5	0.5
0.60	2.5	2.0	1.8	1.8
0.65	17.0	13.0	11.0	9.0
0.70	29.0	23.0	21.0	19.0
0.75	30.0	26.5	25.5	24.0
0.80	30.5	27.5	27.0	26.0
0.90	31.0	28.5	28.0	27.0
1.00	32.0	29.0	28.5	27.5

(Fig. 13)

TABLE 14.

Concentration of stannous Chloride = 13.53×10^{-4}M

pH of ammonium acetate acetic acid buffer = 5.0

Total volume = 20 m.l. Total ionic strength = 0.15

Temperature 30 ± 0.1°C

Applied Potential (Volts)	Current (4×10^{-8} Amperes)		
	0.84% Protein	0.96% Protein	1.08% Protein
0.0	- 29.0	- 29.5	- 26.5
0.05	- 27.5	- 26.5	- 24.5
0.10	- 19.5	- 18.0	- 16.5
0.15	- 7.0	- 5.5	- 5.5
0.20	- 1.4	- 1.0	- 1.0
0.25	- 0.2	- 0.5	- 0.4
0.30	0	0	0
0.40	0.2	0.2	0.2
0.50	0.4	0.4	0.4
0.55	0.5	0.5	0.5
0.60	1.8	1.6	1.5
0.65	8.5	8.0	7.0
0.70	18.0	17.8	15.5
0.75	23.5	22.8	21.0
0.80	25.5	24.8	22.5
0.90	26.5	26.0	23.6
1.00	27.0	26.5	24.4

(Fig. 14)

TABLE 15.

Concentration of stannous Chloride = 13.53×10^{-4}M

pH of ammonium acetate-acetic acid buffer = 5.0

Total volume = 20 m.l. Total ionic strength = 0.15

Temperature 30 ± 0.1°C

Applied Potential (Volts)	Current (4×10^{-8} Amperes)		
	1.2 % Protein	1.68 % Protein	1.8 % Protein
0.0	- 26.5	- 26.5	- 26.5
0.05	- 24.0	- 24.0	- 25.0
0.10	- 16.0	- 15.5	- 15.5
0.15	- 4.5	- 4.5	- 4.5
0.20	- 1.0	- 1.0	- 1.0
0.25	- 0.4	- 0.4	- 0.4
0.30	0	0	0
0.40	0	0	0
0.50	0.2	0.4	0.4
0.55	0.4	0.5	0.5
0.60	2.0	2.0	1.5
0.65	9.0	9.4	8.0
0.70	17.0	17.4	17.0
0.75	21.0	21.5	21.5
0.80	22.5	23.0	23.5
0.90	23.8	24.0	25.0
1.00	24.0	24.5	25.5

(Fig. 15)

T A B L E 16.

Concentration of transfusion gelatin = 0.6% (0.8×10^{-4}M)

pH of Ammonium acetate-acetic acid buffer = 5.0

Total volume = 20 m.l. Total ionic strength = 0.15

Applied Potential (Volts)	Current (8×10^{-9} Amperes)	
	5.22×10^{-4}M Sn^{++}	
	$(id)_o$	(id)
0.0	- 19.0	- 14.0
0.05	- 16.6	- 12.0
0.10	- 15.0	- 9.0
0.15	- 12.8	- 3.5
0.20	- 9.5	- 0.5
0.25	- 4.5	0.8
0.30	- 1.0	1.4
0.40	1.4	1.8
0.50	2.8	2.5
0.55	3.8	3.0
0.60	5.0	3.8
0.65	12.8	8.0
0.70	17.5	11.4
0.75	18.8	12.5
0.80	19.8	13.0
0.90	22.5	14.0
1.00	23.0	15.0

(Fig. 16)

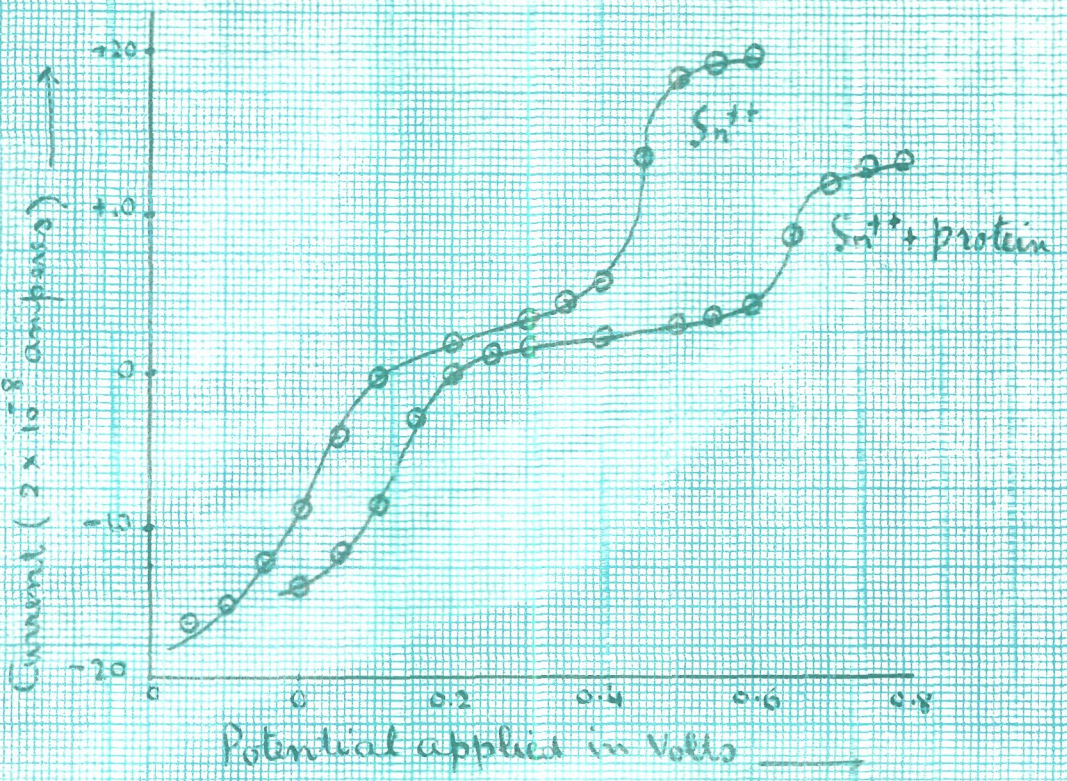

+20
+10
0
-10
-20
Current (2 x 10⁻⁸ amperes)
Sn++
Sn++ + protein
0
0
0·2
0·4
0·6
0·8
Potential applied in Volts

Fig. 16

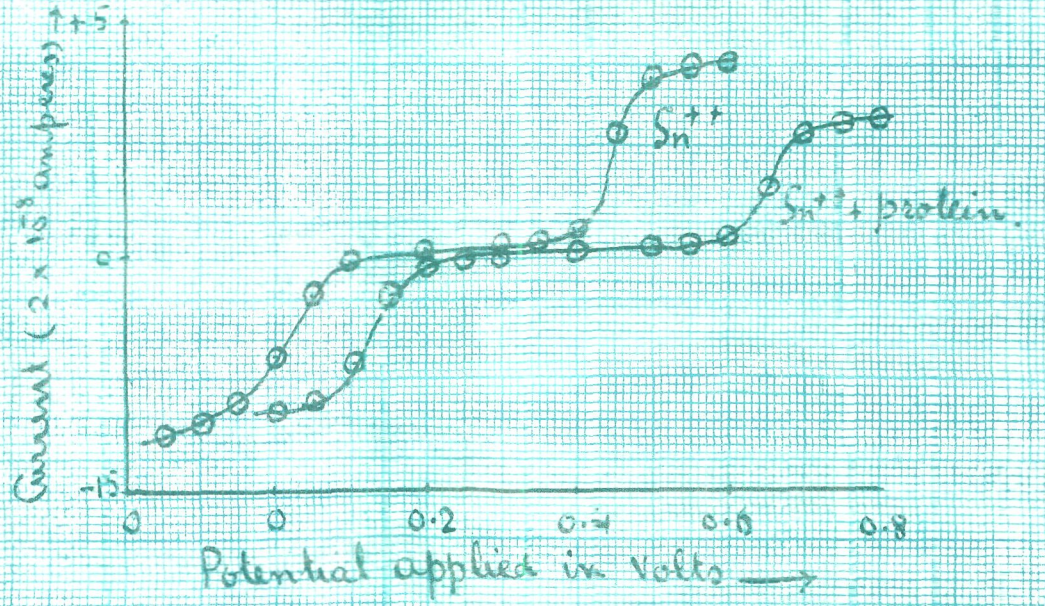

+5
0
-15
Current (2 x 10⁻⁹ amperes)
Sn++
Sn++ + protein.
0
0
0·2
0·4
0·6
0·8
Potential applied in Volts

Fig. 17

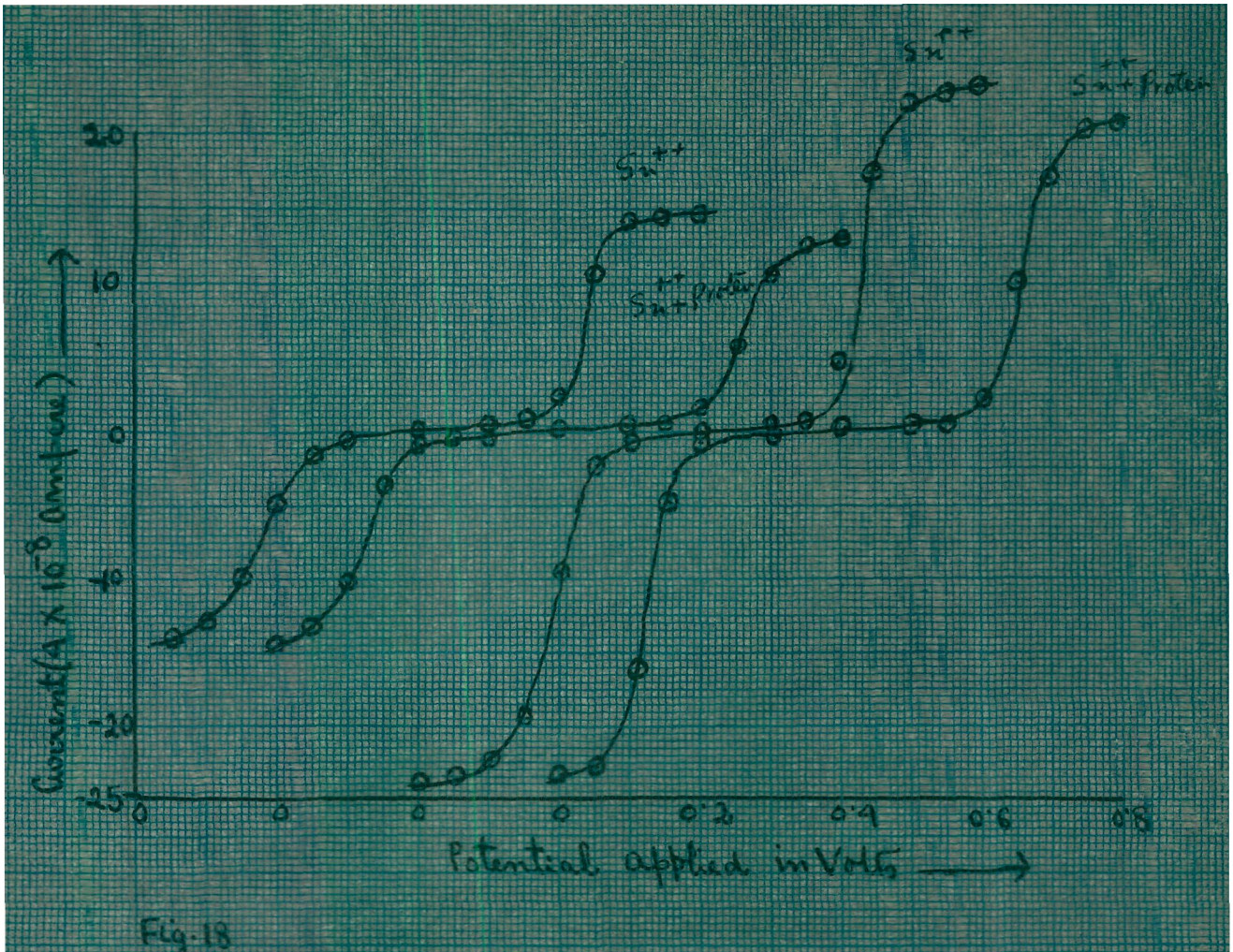

Sn^{++}
Sn^{++} + Protein
20
10
0
-10
-20
-25
Current (A x 10^{-8} Ampere)
0
0
0
0
0·2
0·4
0·6
0·8
Potential applied in Volts

Fig. 18

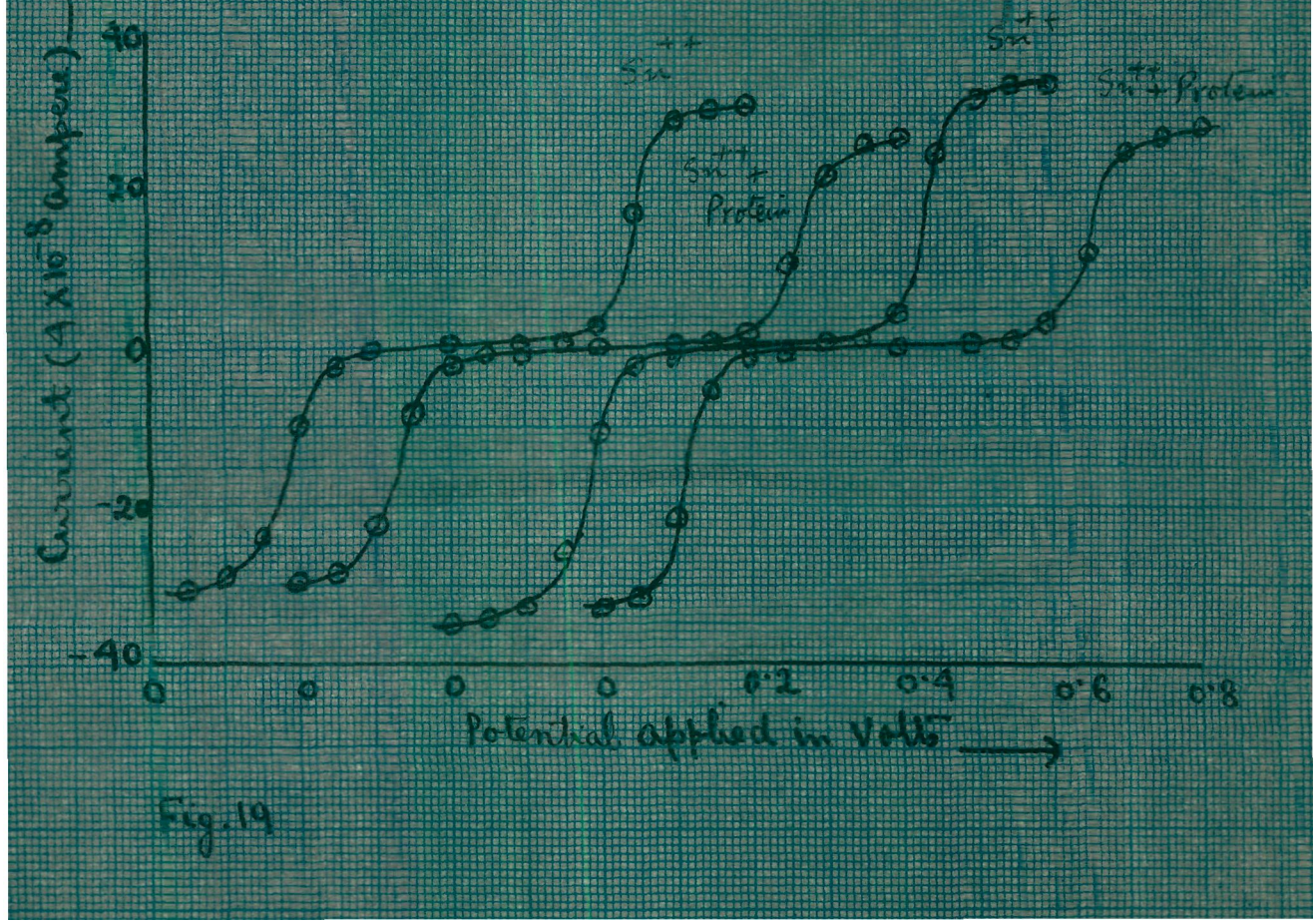

Sn^{++}
Sn^{++} + Protein
40
20
0
-20
-40
Current (A x 10^{-8} Ampere)
0
0
0
0
0·2
0·4
0·6
0·8
Potential applied in Volts

Fig. 19

1.0
0.95
0.9
0.85
0.8
0.75
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
0.55
0.60
0.65
0.70
0.75
0.80
0.85
0.90
0.95
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
2.0
2.1
2.2
2.3
2.4
2.5
2.6
2.7
2.8
2.9
3.0
3.1
3.2
3.3
3.4
3.5
3.6
3.7
3.8
3.9
4.0
4.1
4.2
4.3
4.4
4.5
4.6
4.7
4.8
4.9

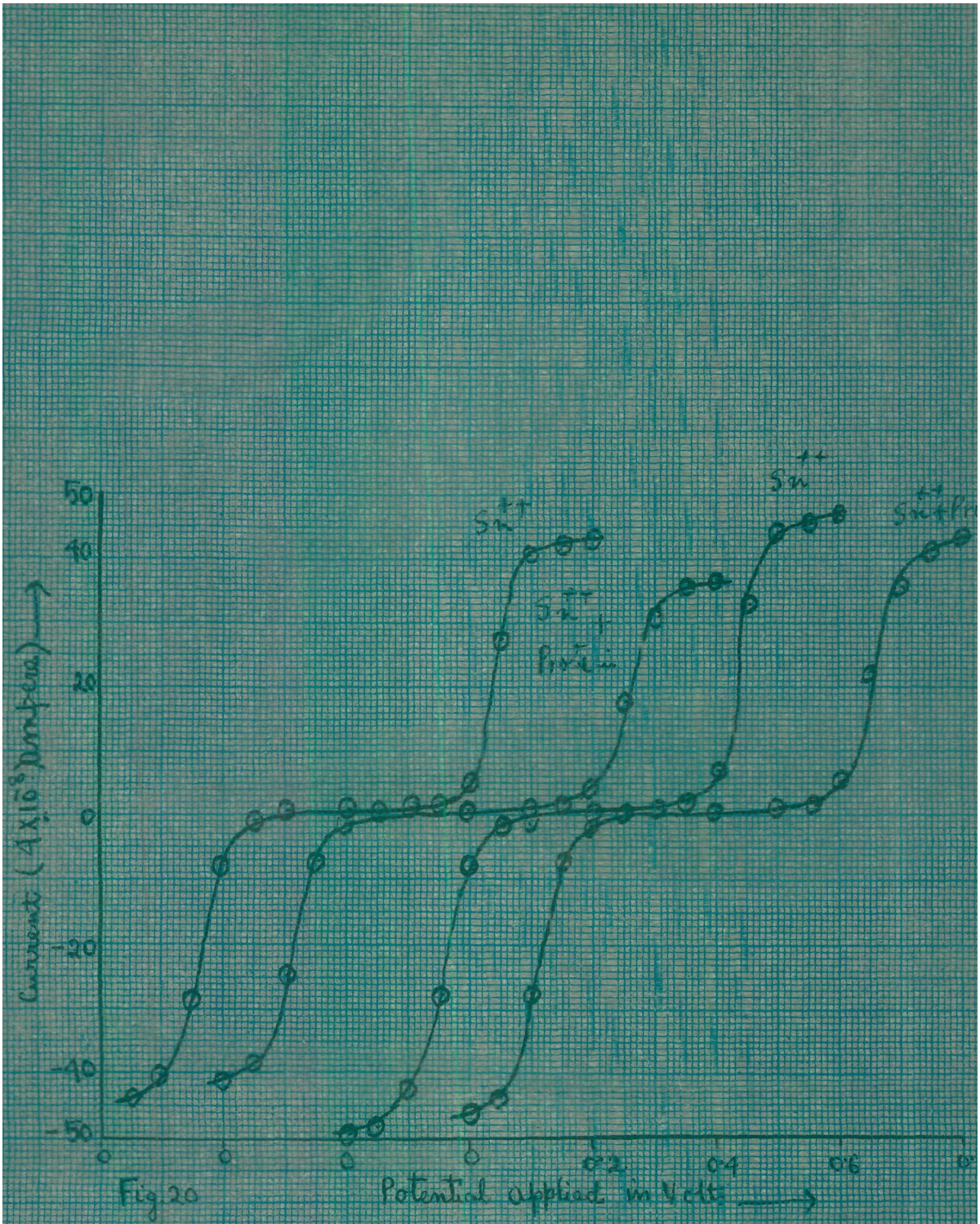

50
10
0
-10
-50
Current (4 x 10⁻⁸ Amp/cm) ⟶
Sn++
Sn++
Sn++
0
0
0
0
0·2
0·4
0·6
Potential applied in Volts ⟶

Fig 20

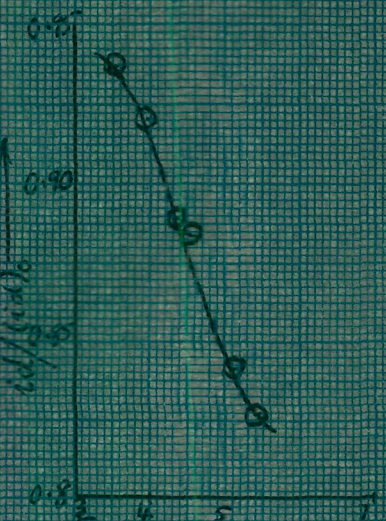
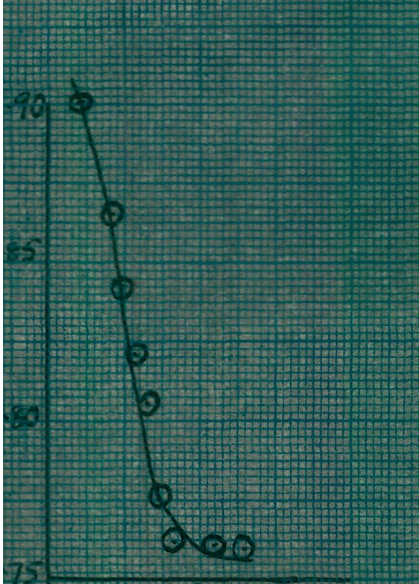


Fig 22A Effect of pH



Protein Concentration (4×10^{-4} M)

Fig 22B Effect of Protein Concentration

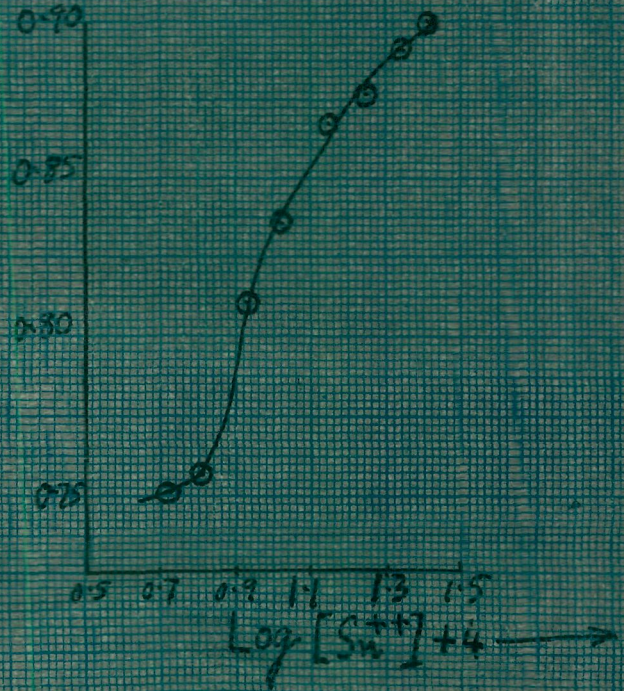


Fig 22C Effect of Metal ion concentration

TABLE 17.

Concentration of transfusion gelatin = 0.6% (0.8×10^{-4}M)

pH of Ammonium acetate-acetic acid buffer = 5.0

Total volume = 20 m.l. Total ionic strength = 0.15

Temperature 30 ± 0.1°C

Applied Potential (Volts)	Current (4×10^{-8} Amperes)	
	$\frac{6.525 \times 10^{-4}\text{M } Sn^{++}}{(id)_o}$	(id)
0.0	- 12.0	- 9.8
0.05	- 11.5	- 9.4
0.10	- 10.8	- 7.0
0.15	- 9.5	- 2.5
0.20	- 6.5	- 0.6
0.25	- 2.5	- 0.2
0.30	- 0.5	0
0.40	0.4	0.2
0.50	0.6	0.5
0.55	0.8	0.5
0.60	1.6	1.2
0.65	8.0	4.6
0.70	11.5	7.8
0.75	12.0	8.5
0.80	12.5	9.0
0.90	13.5	9.2
1.00	14.0	9.5

(Fig. 17)

TABLE 18.

Concentration of transfusion gelatin = 0.6% (0.8×10^{-4}M)

pH Ammonium acetate-acetic acid buffer = 5.0

Total volume = 20 m.l. Total ionic strength = 0.15

Temperature 30 ± 0.1°C

Applied Potential (Volts)	Current (4×10^{-8} Amperes)			
	8.7×10^{-4}M Sn^{++}		10.48×10^{-4}M Sn^{++}	
	$(id)_o$	(id)	$(id)_o$	(id)
0.0	- 14.4	- 14.4	- 24.0	- 23.5
0.05	- 14.0	- 13.5	- 23.5	- 23.0
0.10	- 13.0	- 10.4	- 22.4	- 16.2
0.15	- 10.0	- 3.6	- 19.4	- 5.0
0.20	- 5.0	- 0.8	- 9.5	- 1.0
0.25	- 1.5	- 0.2	- 2.5	- 0.4
0.30	- 0.4	0	- 0.6	0
0.40	0	0.4	0	0.2
0.50	0.4	0.5	0.4	0.4
0.55	0.6	0.5	0.5	0.5
0.60	2.2	1.5	4.6	2.2
0.65	10.5	5.8	17.6	10.0
0.70	14.8	10.5	22.0	17.0
0.75	14.4	12.5	23.0	20.5
0.80	14.5	13.0	23.5	21.0
0.90	15.0	13.5	24.0	21.5
1.00	15.8	13.6	25.0	22.0

(Fig. 18)

TABLE 19.

Concentration of transfusion gelatin = 0.6% (0.8×10^{-4}M)

pH of Ammonium acetate acetic buffer = 5.0

Total volume = 20 m.l. Total ionic strength = 0.15

Temperature $30 \pm 0.1^{\circ}C$

Applied Potential (Volts)	Current (4×10^{-8} Amperes)			
	13.53×10^{-4}M Sn^{++}		17.4×10^{-4}M Sn^{++}	
	$(id)_o$	(id)	$(id)_o$	(id)
0.0	- 31.0	- 29.5	- 35.0	- 33.0
0.05	- 30.5	- 28.8	- 34.0	- 32.0
0.10	- 29.0	- 22.5	- 32.4	- 22.0
0.15	- 23.5	- 8.5	- 26.0	- 6.0
0.20	- 10.0	- 2.0	- 10.5	- 1.0
0.25	- 2.5	- 0.4	- 2.0	- 0.4
0.30	- 0.5	0	- 0.6	0
0.40	0	0.4	0	0
0.50	0.4	0.5	0.4	0.4
0.55	0.6	0.5	0.5	0.5
0.60	2.5	1.8	4.0	2.5
0.65	17.0	11.0	24.5	12.0
0.70	29.0	21.0	31.5	24.0
0.75	30.0	25.5	33.0	26.5
0.80	30.5	27.0	33.5	28.0
0.90	31.0	28.0	33.5	29.0
1.00	32.0	28.5	34.0	29.5

(Fig. 19)

T A B L E 20.

Concentration of transfusion gelatin = 0.6% (0.8×10^{-4}M)

pH of Ammonium acetate acetic acid buffer = 5.0

Total volume = 20 m.l. Total ionic strength = 0.15

Temperature 30 ± 0.1°C

Applied Potential (Volts)	Current (4×10^{-8} Amperes)			
	21.75×10^{-4} M Sn^{++}		26.1×10^{-4} M Sn^{++}	
	$(id)_o$	(id)	$(id)_o$	(id)
0.0	- 45.0	- 42.0	- 50.0	- 47.0
0.05	- 44.0	- 40.0	- 49.0	- 45.0
0.10	- 41.0	- 26.5	- 43.0	- 28.0
0.15	- 29.0	- 8.0	- 28.0	- 8.0
0.20	- 9.0	- 1.0	- 8.5	- 1.2
0.25	- 1.8	0	- 2.0	0
0.30	- 0.5	0	- 0.5	0
0.40	0	0.2	0	0.2
0.50	0.4	0.5	0.5	0.4
0.55	0.5	0.5	1.0	0.6
0.60	4.0	3.5	6.0	4.8
0.65	26.0	17.0	31.8	21.0
0.70	40.0	29.5	43.5	35.0
0.75	41.5	35.0	45.0	41.0
0.80	42.5	37.0	46.8	43.0
0.90	43.0	38.0	47.5	44.0
1.00	43.5	38.0	47.5	44.0

(Fig. 20)

RESULTS AND DISCUSSION

Assuming that the decrease in diffusion current is solely due to complex ion formation between the metal and the protein, the following procedure may be employed to evaluate the binding data. The Ilkovic equation,

$$(id)_o = \alpha Co \text{ - - - - - - (2)}$$

where Co is the concentration of metal ion and α is the Ilkovic constant, takes the form

$$(id) = \alpha (C_F + kC_b) \text{ - - - - - (3)}$$

(where C_F is the free metal concentration, C_b the concentration of bound metal ions and k is the fractional coefficient,), in case of complex ions due to the two factors viz., (i) the reduction of free metal ion at D.M.E. and (ii) the reduction of bound metal ions (which is however very slow) contributing to the total diffusion current.

Combining equation 2 and 3.

$$(id)/_{(id)_o} = \frac{C_F + kC_b}{C_o} \text{ - - - - - (4)}$$

Since $Co = C_F + C_b$, equation (4) can be used to estimate C_b, the concentration of bound metal ions, provided $(id)/_{(id)_o}$ and k are known.

The value of $(id)/_{(id)_o}$ may be determined by measuring the diffusion current of metal ions in presence and absence of protein, for a given system, while the constant k, which is pH dependent and characteristic of a system may be obtained by plotting the ratio $(id)/_{(id)_o}$ against pH (k is the limiting value of $(id)/_{(id)_o}$). From the values of C_b thus obtained one can find VM, the number of metal ions bound per protein molecule from the following relationship

$$VM = \frac{C_b}{P} \quad \text{- - - - - - - - - (5)}$$

where P is total molar concentration of protein.

Furthermore the intrinsic association constant for the complex may be determined as follows[43]. Let θ be the fraction of the site in a protein molecule available for binding with A, the unbound fraction is than $(1-\theta)$, and at equilibrium

$$\frac{\theta}{(1-\theta)} = k\, C\, A \quad \text{- - - - - - - - (6)}$$

where C_A is the free concentration of A. If we assume that two substances A and B compete for a common binding site, if so, θ_A, θ_B and $(1-\theta_A-\theta_B)$ would represent the fraction of sites combining with A or B or the unreacted part, then at equilibrium

$$K_A C_A = \frac{\theta_B}{(1 - \theta_A - \theta_B)} \quad \text{- - - - - (7)}$$

$$K_B C_B = \frac{\theta_B}{(1 - \theta_A - \theta_B)} \quad \text{- - - - - - (8)}$$

If there are 'n' number of such identical and completely independent sites, and V_A and V_B represent the number of such sites occupied by A and B, then neglecting the electrostatic interaction factor:

$$K_B = \frac{V_B}{(n - V_A - V_B)\, C_B} \quad \text{- - - - - - (9)}$$

where K_B is the intrinsic association constant for the combination of B with the macromolecule. If we assume a competition between metal and hydrogen ion for a common site then equation (9) becomes

$$K_M = \frac{V_M}{(n - V_H - V_M)\, C_M} \quad \text{- - - - - - (10)}$$

the calculation of intrinsic association constant by means of equation (10) entails a knowledge of V_M, n, V_H and C_M. V_M and C_M may be determined polarographically while the values of V_H and n are available from literature[44].

The results on the interaction of lead and tin with transfusion gelatin, based on the above calculation are summarised in the following tables.

TABLE 21.

Lead - transfusion gelatin system

A - Effect of pH:

Concentration of transfusion gelatin = 0.6%(0.8×10^{-4}M)

Concentration of lead nitrate = 0.5×10^{-3}M

pH	$(id)_o$ (4×10^{-8} Amp)	$(id)_8$ (4×10^{-8} Amp)	$(id)/(id)_o$	C_b ($\times 10^{-3}$M)	V_M
3.72	36.8	33.8	0.918	0.136	1.7
4.45	36.8	33.5	0.910	0.150	1.9
4.80	36.8	33.2	0.902	0.163	2.0
5.20	36.8	32.0	0.869	0.218	2.7
5.57	36.8	31.4	0.853	0.245	3.1

Concentration of transfusion gelatin = 0.6%(0.8×10^{-4}M)

Concentration of lead nitrate = 0.4×10^{-3}M

pH	$(id)_o$ (4×10^{-8} Amp)	$(id)_8$ (4×10^{-8} Amp)	$(id)/(id)_o$	C_b $\times 10^{-3}$M	V_M
5.0	33.0	26.8	0.81	0.253	3.2
5.95	32.5	25.7	0.790	0.280	3.5
6.35	32.0	25.0	0.780	0.293	3.6
6.80	32.0	25.0	0.780	0.293	3.6

(Fig. 21 A)

N.B.: The diffusion current of lead ion at different pH values in the absence of protein remained constant upto pH 5.5 and then decreased with increase in pH. This was attributed to the existence of aqua-complexes[26] at higher pH range. This fact was taken into consideration when computing the values of $(id)/(id)_o$

B - Effect of protein concentration

Concentration of lead nitrate = 0.5×10^{-4}M

pH 5.57

Concentration of protein $\times 10^{-4}$M	$(id)_o$ (4×10^{-8}Amp.)	(id) (4×10^{-8}Amp.)	$(id)/(id)_o$	C_b (10^{-3}M)	VM
0	36.8	-	-	-	-
0.4	36.8	33.6	0.914	0.143	3.5
0.6	36.8	32.6	0.885	0.191	3.2
0.8	36.8	31.4	0.853	0.245	3.1
1.2	36.8	30.0	0.814	0.310	2.6
1.4	36.8	27.4	0.743	0.428	3.0
1.6	36.8	26.8	0.728	0.453	2.8
1.8	36.8	26.1	0.709	0.485	2.7
2.0	36.8	25.8	0.704	0.493	2.5

(Fig. 21B)

C - Effect of metal ion concentration

Comcentration of protein = 0.6 % (0.8×10^{-4}M)

pH 5.57

Concentration of P_b(11) $\times 10^{-3}$M	$(id)_o$ (4×10^{-8}Amp.)	(id) (4×10^{-8}Amp.)	$(id)/(id)_o$	C_b ($\times 10^{-3}$M)	VM
1.0	66.0	59.0	0.893	0.356	4.4
0.75	52.5	45.5	0.866	0.336	4.1
0.50	36.0	30.7	0.853	0.245	3.1
0.35	25.0	21.0	0.840	0.186	2.3
0.20	14.0	10.2	0.725	0.183	2.2
0.10	6.0	4.2	0.700	0.100	1.2

(Fig. 21C)

As stated earlier, the appreciable reduction in diffusion current of metal ions in presence of protein (amounts larger than required for suppression of the maxima) has been ascribed to; (i) the probable complex formation between metal and protein; (ii) adsorption and (iii) viscosity effects. Since the present investigations were carried out at pH 5.5 and above and every care was taken to keep the protein in its native state, the influence of non-specific factors like adsorption and viscosity can very well be ruled out, and the relative depression in diffusion current can, therefore, be taken as a true measure of metal-protein combination. This observation combined with the fact, that the value of V_M, the number of metal ions bound per protein molecule, remained the same at all protein concentration and that a limiting value of $(id)/_{(id)_o}$ is realised by changing either the metal:protein ratio or the pH provide further confirmation, that lead gets bound to transfusion gelatin.

From table (21.A) it appears that although there is a small reduction of diffusion current, in the pH range 3.72 to 4.8, a large decrease is observed beyond pH 5.0 (pH 4.8 to 5.9), with a subsequent increase in C_b. These results indicate the uptake of metal ion increases more rapidly in the vicinity of

pH 5.0. At pH 5.5 all the 84 carboxyl groups of transfusion gelatin are deprotonated, and are thus available for binding metal ions. These results lead to the conclusion that carboxyl groups offer principal site for the binding of plumbous ions. Assuming such an interaction the intrinsic association constant, calculated by applying equation (10) comes out to be 1.81 (log K = 1.81), and free energy change $-\Delta F$ = 2.593 KCal.

One of the interesting question on the combination of metals with protein is: can the affinity be explained on the basis of combining power of amino acids residues? If the reactivity of carboxylate ion in transfusion gelatin is taken to be roughly equivalent to that of acetate ion, (log K for for lead acetate 2.7)[45] than it becomes clear that plumbous ions have lesser affinity for carboxylate ion in transfusion gelatin. Such a behaviour is not unlikely in view of the competition which exists between the carboxylate ion of the protein, and the anions (nitrate ions) of the supporting electrolyte. The effective concentration of carboxylate ion at pH 5.5 is 0.0068M (taking the molecular weight of transfusion gelatin 75,000 and 84 carboxyl groups are completely dissociated) in 0.8×10^{-4}M protein, whereas the effective concentration of supporting

electrolyte is 0.15M. Since lead has a complexing tendency with nitrate ($\log K = 1.1$[45]) therefore, it may be assumed that such a competition exists. Furthermore electrostatic effect may be operative for second incoming lead ion when an adjacent carboxyl group has already been occupied by a metal ion.

At pH higher than 5.5, imidazole groups from histidine residue lose their proton and are thus available for the binding of metal ions, and a further drop in diffusion current would be expected with the increase in pH. The relatively small decrease in diffusion current observed in the pH range 5.9 - 6.8 give little or no evidence for the binding of lead to the imidazole groups.

Tin-transfusion gelatin system

T A B L E 22.

A-Effect of pH

Concentration of Sn(ii) = 13.53×10^{-4}M

Concentration of transfusion gelatin = 0.96×10^{-4}M

pH	$(id)_o$ (4×10^{-8}Amp)	(id) (4×10^{-8}Amp)	$(id)/(id)_o$	C_b $\times10^{-4}$M	V_M
3.5	31.0	29.0	0.935	3.700	3.8
3.9	31.0	28.5	0.920	4.510	4.6
4.3	31.0	27.5	0.888	6.314	6.4
4.6	31.0	26.8	0.886	7.554	7.8
5.0	31.0	26.0	0.840	9.020	9.2
5.3	31.0	25.5	0.825	9.865	10.3

(Fig. 22 A)

B-Effect of protein concentration

Concentration of Sn(ii) = 13.53×10^{-4}M

pH = 5.0

Concentration of protein $\times10^{-4}$M	$(id)_o$ (4×10^{-8}Amp)	(id) (4×10^{-8}Amp)	$(id)/(id)_o$	C_b ($\times10^{-4}$M)	V_M
0.40	31.0	27.5	0.900	5.64	14.1
0.80	31.0	26.8	0.866	7.55	9.4
0.96	31.0	26.0	0.840	9.02	9.3
1.12	31.0	25.5	0.820	10.15	9.0
1.28	31.0	25.0	0.806	11.27	8.8
1.44	31.0	24.0	0.77	12.96	9.0
1.60	31.0	23.5	0.76	13.53	8.4
2.24	31.0	23.5	0.76	-	-
2.40	31.0	23.5	0.76	-	-

(Fig. 22 B)

C-Effect of metal ion concentration

Concentration of protein = 0.8×10^{-4}M

pH = 5.0

Concentration of Sn(ii) x 10^{-4}M	$(id)_o$ ($4x10^{-8}$Amp)	(id) ($4x10^{-8}$Amp)	$(id)/(id)_o$	C_b ($x10^{-4}$M)	V_M
5.220	11.5	8.7	0.750	5.22	5.5
6.525	12.0	9.0	0.756	6.525	8.0
8.700	15.0	12.0	0.810	6.889	8.6
10.440	24.0	20.5	0.835	7.178	8.9
13.530	31.0	26.8	0.866	7.554	9.4
17.400	33.5	28.5	0.875	9.061	11.3
21.750	42.0	38.0	0.890	9.960	12.5
26.100	46.0	43.5	0.900	10.870	13.5

(Fig. 22 C)

The marked decrease in diffusion current of stannate ions, in presence of increasing amount of transfusion gelatin, coupled with the fact that a limiting value of $(id)/(id)_o$ (0.76 in the present case) is realised by changing the metal: protein ratio; indicates the uptake of metal ions by the specific potential sites of the protein molecule. The factors like viscosity changes and adsorption which are also supposed to be responsible for such behaviour, may be ruled out in view of the fact that, the protein has been kept in the native state and more over the studies have

been performed above iso-electric point. Furthermore $(id)/(id)_o$ would have increased with increase in pH if adsorption were the only factor. Hence the observed decrease in diffusion current, with increase in pH may be taken as a measure of the metal protein combination.

Fig.(22 A), illustrates the characteristic effect of pH on the extent of binding of Sn(ii) to transfusion gelatin. The value of V_M, the number of metal ions bound per protein molecule, ranges between 4 to 10 in the pH range 3.5 to 5.3, where the carboxyl groups are likely to be the principal sites for the binding of metal ions. The upper limit of the curve leads one to conclude that the groups other than carboxyl (possibily imidazole) are not involved in complex ion formation. This, however, would not be strictly tested as in the case of plumbous ion since the Sn(ii) is reduced irreversibily in the neutral pH region.

The intrinsic association constant of Sn(ii)-carboxyl groups of transfusion gelatin as calculated from Scatchard's equation comes out to be 1.94 (log K = 1.94), when an average value of 9 is taken for V_M at total metal concentration 13.53×10^{-4} M, and free energy change of the reaction is $\Delta F = -2.703$ KCal.

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(b) CHAPTER 2.

pH-metric studies on the interaction of Copper, Zinc, Cadmium, Lead, Cobalt and Nickel with transfusion gelatin.

I N T R O D U C T I O N

pH titrations have proved to be of immense value in studying metal-protein interaction, since the latter behave as multivalent electrolyte and offer a large number of ionisable groups in the different pH range. Although the technique has not been fully utilised in the study of metal-protein complexes, it has been almost completely worked out for various proteins; in terms of hydrogen ion equilibria. Since their study is based on simple pH-metric titration, and one has got to come across complexities, evidenced in Bjerrum's method, which is mainly used for determining metal ion binding. Beside the simplicity in technique, the results on hydrogen ion equilibria furnish valuable information, regarding the various characteristics[1-3] of the protein molecule under study; viz., number of ionisable groups; pK-values; configurational changes and average net charge.

Investigations on the metal ion binding of proteins remain purely of qualitative nature, unless data on acid base binding capacities of protein are not made available.

A knowledge of the development made in this direction, therefore, becomes of primary importance for workers in the field of metal-protein complexes. A short resume of the work carried out on hydrogen ion binding is therefore, worth describing. The studies on transfusion gelatin-metal interaction[4,5] reported from this laboratory were preceded by the extensive work on hydrogen ion equilibria[6].

The present chapter deals with the studies on the binding of copper, zinc, cadmium, cobalt, nickel and lead with transfusion gelatin employing the pH metric method. The results have given evidence for the binding of these metal ions at different sites of transfusion gelatin - a study which has not hitherto been carried out with fibrous protein. It may, however, be added that attempts in this direction have been made by few workers[7,8].

Acid - Base - binding

Burgarozky and Liebermann[9] for the first time, reported the results on the electromotive force measurements on the acid-base-binding capacities of the proteins. Since then a number of workers notably, Robertson[10], Rohonyi[11], Manabe and Matula[12], Blasel and Matula[13], Pauli[14] and Lloyd and Mayes[15] have worked on these lines. Conductometric[16,17] and Cyroscopic[18] methods

were also tried, but with little success. Van Slyke[19], Orying and Pauli[20], Bracewell[21] and Izaquirre[22] tried to explain the phenomena of acid- and base-binding on the basis of physical adsorption, and held the opinion that acids and bases are adsorbed on the large surface offered by the protein molecule.

A purely chemical view point was, however, put forwarded by Loeb[23] in 1922, who demonstrated that acid binding with protein took place in strictly stoichiometric ratio[24,25]. Two years later, another notable contribution in this field was made by Linderstrom-Lang[26], who gave an adequate theoretical interpretation of the phenomenon on the basis of Debye-Hukel theory. His treatment was extended by Cannan, Kibrick and Palmer[27,28], Kirkwood[29], Scatchard[30], and Tanford[1,31], the work of these authors helped in providing a theoretical basis for the binding of small ions by proteins in terms of equilibrium constants.

Titration Curves

A titration curve of protein, which resembles with the titration curve of a poly-basic acid, may be divided into a number of overlaping regions, if the ionising groups being titrated are assumed to be intrinsically identical. Each region is then taken to correspond to the titration of these intrinsically identical groups.

The α Carboxyl groups at the end of the peptide chain, would be intrinsically identical forming one region of the titration curve. This is followed by another region due to β-Carboxyl groups from aspartyl residue and γ-Carboxyl groups from glutamyl residue. The next one is due to imidazole groups (from histidine residue) and then ϵ-amino groups (from lysyl residue), phenotic groups (from tyrosyl residue) and finally the guanidino groups (from arginyl residue). The number of hydrogen ions dissociated or combined per protein molecule, would give an over all titration curve[1] composed of different regions, corresponding to the dissociation ranges of the identical groups in the protein.

The number of ionisable groups of various proteins such as Haemoglobin[32,34], serum albumin[31], egg albumin[27], β-lactoglobin[28,35], lysozyme[36], Insulin[7,37], Conalbumin[38], Ribonuclease[39], Woolkeratin[40], Myosin[41], Casein, α-, β-, and γ-casein[42], Collagen[43], gelatin[44-55] and Metmyoglobin[56], have been determined by pH-metric method.

Interaction of metals with proteins

Tanford and Epstein for the first time employed the titration curves for the study of metal-protein interactions[7]. The basic concept on which hydrogen ion titration data may be utilized to elucidate the nature

of the binding of ions other than hydrogen ion, was laid down by Tanford[57], and deserves some mention here.

It is a well known fact that metal ions form complexes in solution with basic substances, that is with substances which combine with hydrogen ion. The reaction is, therefore, a competitive one and may be represented as:

$$M^{-z} + NH_3 \rightleftharpoons M(NH_3)^{-z}, M(NH_3)_2^{-z} \quad \ldots\ldots (1)$$

the equilibrium in such a reaction, may be measured indirectly by observing the displacement of corresponding hydrogen ion equilibrium.

$$NH_4^{-} \rightleftharpoons NH_3 + H^{+} \quad \ldots\ldots\ldots (2)$$

This method has been extensively used by J. Bjerrum[58]. The same principle may apply to the combination of metals to more complex molecule. A competition between metal and hydrogen ion for a common site, necessitates that the metal ion can combine only with the basic (deprotonated) functional groups. The uptake of metal ions will, however, shift the hydrogen ion equilibria in the direction of the basic side of the functional groups. The hydrogen ions thus released, results in a decrease in pH.

It is customary to express the hydrogen ion equilibria in terms of dissociation constant. If it is

assumed that all ionisable groups of a given type are intrinsically identical, the degree of dissociation of such groups at any pH is given by[57],

$$\log \frac{x_i}{(1 - x_i)} = pH - (pK\ int) - 0.868\ ZW \quad . \quad . \quad . \quad (3)$$

where x_i is the degree of dissociation of groups of the ith kind, (pKint) is their intrinsic dissociation constant at the ionic strength used, Z is the net charge on the protein molecule at a given pH and W is an empirical parameter which depends (for a particular temperature and a given ionic strength) on the size and shape of the protein molecule. If n_i is the total number of intrinsically identical ionisable groups of ith type in the protein molecule and r_i is the number of such groups dissociated at a given pH, than equation (3) may be written as

$$\log \frac{r_i}{(n_i - r_i)} = pH - (pK_{int}) \pm 0.868\ ZW \quad . \quad . \quad . \quad (4)$$

The value of r_i, the number of hydrogen ions dissociated per protein molecule, at any pH, can be read off from the titration curve. The above equation represents a titration curve in absence of metal ion. In presence of metal ions, a fraction of acidic groups will be removed from participation in hydrogen ion equilibria by metal binding. Therefore, it is possible to calculate the binding data directly from the difference

in hydrogen ion titration curves in the presence and absence of metal ions, without a direct determination of V_M. Incidentally this method is identical in principle with Bjerrum's method.

EXPERIMENTAL

Apparatus and Technique:

pH-measurements were carried out by means of Backman Model G, pH - meter, using glass electrode. The pH meter was standardised against 0.05M potassium acid phthalate (pH 4.0), standard Backman buffer (pH 7.0) and 0.05M sodium borate (pH 9.2), All measurements were carried out, keeping the cell in water thermostate (Townson and Mercer Croydon) at t $\pm$ 0.1°C. Nitrogen (purified by passing through alkaline pyrogallol and chromous chloride) was passed slowly for about 10 to 15 mts to ensure inert atmosphere.

Solutions and reagents

Transfusion gelatin(concentration of 6% molecular weight 75,000) was used throughout these investigations.

Stock solution of hydrochloric acid was prepared from A.R. reagent grade sample and the strength determined

by usual method. Carbonate free potassium hydroxide was prepared according to Kolthoff[59] and its concentration determined by pH metric titration with standard potassium acid phthalate solution. A.R. samples of potassium chloride, potassium nitrate, potassium acid phthalate and sodium borate were dissolved in triply distilled water (distilled in all glass apparatus) to get their respective solutions. Chemically pure (E.Merck) samples of lead nitrate cupric chloride, zinc sulphate, cadmium sulphate, cobalt chloride and nickel chloride were used as source of metal ions. Lead, cadmium and zinc content were determined against EDTA and E.Black T as titrant and indicator respectively where as copper, cobalt and nickel content were found out by titrating them against EDTA using Murexide as indicator[60].

Procedure

Varying amounts of hydrochloric acid (0.7966 $\frac{N}{10}$), viz., 1.6, 1.0, 0.8, 0.7, 0.5, 0.3, 0.1, 0.02 and 0 m.l. and those of potassium hydroxide (0.5575 $\frac{N}{10}$) viz., 0.2 , 0.3, 0.4, 0.6, 1.0, 1.1 and 1.5 m.l. were taken in different pyrex test tubes. Twelve such sets were arranged and in each case total volume made upto 10 m.l. by the addition of the requisite amounts of potassium chloride (to keep the ionic strength at 0.15) distilled water and the respective reagents given below:

Set No.	Reagents
1.	-
2.	1 m.l. transfusion gelatin (6% concentration)
3.	2 m.l. cupric chloride (0.01M)
4.	2 m.l. cupric chloride + 1 m.l. transfusion gelatin
5.	1 m.l. zinc sulphate (0.01M)
6.	1 m.l. zinc sulphate + 1 m.l. transfusion gelatin
7.	1 m.l. cadmium sulphate (0.0088M)
8.	1 m.l. cadmium sulphate + 1 m.l. transfusion gelatin
9.	1 m.l. cobalt chloride (0.01M)
10.	1 m.l. cobalt chloride + 1 m.l. transfusion gelatin
11.	1 m.l. nickel chloride (0.01008M)
12.	1 m.l. nickel chloride + 1 m.l. transfusion gelatin

The pH of the solutions were recorded immediately after mixing and after 24 hours. No detectable change in pH occured after a considerable laps of time, All measurements were carried out at 25°C.

The pH - values for lead transfusion gelatin mixtures were taken at 30°C and the following sets were prepared. Varying amounts of hydrochloric acid (0.8781 $\frac{M}{10}$) viz., 1.4, 0.9, 0.7, 0.6, 0.4, 0.2, 0.1, 0.08 and 0 m.l. were mixed with 2 m.l. transfusion gelatin.(3%) in different test tubes keeping the total volume at 10 m.l. and the ionic strength at 0.15. The pH of each solution was recorded immediately after mixing and then again after 24 hours. The values were found to be the same

in both cases. The measurements were repeated without lead ion but maintaining the same conditions.

Experiments were also performed following Tanford to show the reversible nature of hydrogen ion equilibria in presence of metal ions[37].

TABLE 1.

Concentration of transfusion gelatin = 0.6 %

Total Volume 10 m.l. Total ionic strength = 0.15

Temperature 25°C

HCl ($\frac{0.7966\ N}{10}$) added ml	pH - values			
	With acid or Alkali	With Protein	2.0×10^{-3} M Cu^{+2}	2.0×10^{-3} M Cu^{+2} Protein
1.6	1.95	2.00	1.96	2.19
1.0	2.16	2.63	2.16	2.60
0.8	2.25	3.01	2.56	2.95
0.7	2.32	3.40	2.34	3.25
0.5	2.45	3.90	2.46	3.60
0.3	2.70	4.40	2.71	4.00
0.1	3.10	5.05	3.08	4.55
0.02	3.80	5.30	3.80	4.90
0	6.40	6.80	5.60	5.40
KOH ($\frac{0.5575\ N}{10}$) added m.l.				
0.2	10.88	7.5	5.9	5.8
0.3	-	-	6.8 ppt.	6.78
0.4	11.18	8.22	8.4 "	7.55
0.6	11.35	8.80	10.10 "	8.00
1.0	-	9.50	-	9.00
1.1	11.50	10.20	-	9.50
1.5	11.82	11.20	-	10.60

(Fig. 1.)

TABLE 2.

Concentration of transfusion gelatin = 0.6 %

Total Volume 10 m.l. Ionic strength = 0.15

Temperature 25°C

KCl(0.7966 $\frac{N}{10}$) added m.l.	pH - values			
	1.0×10^{-3}M Zn^{+2}	1.0×10^{-3}M Zn^{+2} Protein	0.88×10^{-3}M Cd^{+2}	0.88×10^{-3}M Cd^{+2} Protein
1.6	1.95	2.20	1.95	2.20
1.0	2.16	2.61	2.15	2.61
0.8	2.26	3.00	2.24	2.98
0.7	2.33	3.27	2.32	3.30
0.5	2.45	3.72	2.45	3.78
0.3	2.72	4.28	2.71	4.30
0.1	3.10	4.90	3.10	4.92
0.02	3.80	5.00	3.81	5.10
0	6.14	6.10	7.0	6.42
KOH (0.5575 $\frac{N}{10}$) added m.l.				
0.2	7.2 ppt.	6.90	7.8 ppt.	7.20
0.4	9.0 "	7.80	8.9 "	8.00
0.6	11.0 "	8.20	10.0 "	8.50 ppt.
1.0	-	9.30	-	9.20 "
1.1	-	10.00	-	9.90 "
1.5	-	10.98	-	10.50 "

(Fig. 2)

TABLE 3.

Concentration of transfusion gelatin = 0.6 %

Total volume 10 m.l. Ionic strength = 0.15

Temperature 25°C

HCl($\frac{0.7966\ N}{10}$) added m.l.	pH - values			
	1.0×10^{-3}M Co^{+2}	1.0×10^{-3}M Co^{+2} Protein	1.008×10^{-3}M Ni^{+2}	1.008×10^{-3}M Ni^{+2} Protein
1.6	1.94	2.20	1.94	2.20
1.0	2.16	2.60	2.16	2.60
0.8	2.25	2.98	2.26	3.00
0.7	2.31	3.30	2.30	3.32
0.5	2.45	3.70	2.46	3.75
0.3	2.70	4.25	2.71	4.28
0.1	3.12	4.85	3.10	4.90
0.02	3.80	5.00	3.80	5.00
0	7.60	6.48	7.80	6.45
KOH($\frac{0.5575\ N}{10}$) added m.l.				
0.2	8.28 ppt.	7.38	8.40ppt	7.12
0.4	8.82 "	8.05	9.00 "	8.10
0.6	11.15 "	8.25 ppt.	10.28 "	8.65 ppt.
1.0	-	9.40 "	-	9.40 "
1.1	-	9.90 "	-	10.20 "
1.5	-	10.55 "	-	10.88 "

(Fig. 3)

TABLE 4.

Concentration of transfusion gelatin = 0.6 %

Total volume 10 m.l. Ionic strength = 0.15

Temperature 30°C

HCl (0.8781 $\frac{N}{10}$) added m.l.	pH - values	
	With Protein	With 0.4×10^{-3} M Pb^{+2} Protein
1.4	2.23	2.22
0.9	2.70	2.65
0.7	3.15	3.08
0.6	3.50	3.30
0.4	4.00	3.70
0.2	4.60	4.30
0.1	4.90	-
0.08	-	4.70
0.0	5.56	5.58

(Fig. 4)

RESULTS AND DISCUSSION

The value given in column 2 of the tables (5 to 11) are converted into free hydrogen or hydroxyl ions, assuming[61] that a_{H^+} or a_{OH^-} depends only upon the non protein constituent of the solution even in presence of protein. The pH is given as

$$pH = -\log a_{H^+} \quad r_{H^+} = \log a_{OH^-} \quad r_{OH^-}/KW$$

the activity coefficients of hydrogen and hydroxyl ions at extreme acid and basic ranges are taken from Tanford[31], and the values of KW from those obtained by Harned and Co-workers[62] (1.008 and 1.47×10^{-4} at 25 and 30°C respectively). The difference between the added and free hydrogen ions gives the number of hydrogen ions bound to the protein. From this r, the number of hydrogen ions dissociated per protein molecule is evaluated both in presence and absence of metal ions. Thus, titration curves for the systems, copper-transfusion

gelatin, zinc-transfusion gelatin, cadmium-transfusion gelatin, cobalt-transfusion gelatin, nickel-transfusion gelatin, lead-transfusion gelatin and transfusion gelatin alone are obtained by plotting 'r' against pH.

The metal ions have a pronounced effect on the titration curves of transfusion gelatin, the titration curves show a shift towards the lower pH region. From this shift, the number of displaced hydrogen ions are computed for the above systems. If Gurd and Murray's assumption[63] - one to one binding of lead to the carboxyl groups of serum albumin - be extended to the present systems, the number of hydrogen ions displaced in presence of metal ions, directly determines V_M, the number of metal ions bound per protein molecule.

The intrinsic association constants for the combination of Cu, Zn, Cd, Co, Ni and Pb have been calculated as usual applying Scatchard equation.[30]

TABLE 5.

Concentration of transfusion gelatin = 0.6 % (0.8×10^{-4} M)

Ionic strength = 0.15 Temperature 25°C

H^+ added moles/ $L.10^3$	pH	Free H^+ moles/ $L.10^3$	Bound H^+ moles/ moles	Moles of H^+ dissociated per mole protein
12.745	2.20	7.1610	70.0	0
7.966	2.63	2.6120	67.0	3
6.373	3.01	1.0890	66.0	4
5.576	3.40	0.3980	64.0	6
3.983	3.90	0.1260	48.0	22
2.3898	4.40	0.0398	29.0	41.
0.7966	5.05	0.0089	10.0	50
0.1593	5.30	0.0001	2.0	68
0	6.80	0	0	70

Base(OH^-) added moles/$L.10^3$	pH	Free OH^- moles/ $L.10^3$	Bound OH^- moles/ $L.10^3$	Moles of H^+ dissociated per mole protein
1.1150	7.50	0.0003	14.0	84
2.2300	8.22	0.0016	28.0	98
3.3450	8.80	0.0064	42.0	112
5.5750	9.50	0.0340	69.0	139
6.1325	10.20	0.1704	75.0	145
8.3625	11.20	1.7040	83.0	153

(Fig. 5A)

TABLE 6.

Concentration of transfusion gelatin = 0.6% (0.8×10^{-4}M)

Concentration of Cupric Chloride = 2.0×10^{-3}M

Ionic strength = 0.15 Temperature 25°C

H^+ added moles/ $L.10^3$	pH	Free H^+ moles/ $L.10^3$	Bound H^+ moles/mole protein	Moles of H^+ dissociated per mole protein
12.7450	2.19	7.3280	68.0	2.0
7.9660	2.60	2.7990	65.0	5.0
6.3730	2.95	1.2500	64.0	6.0
5.5760	3.25	0.5623	63.0	7.0
3.9830	3.60	0.2512	47.0	23.0
2.3898	4.00	0.1000	29.0	41.0
0.7966	4.55	0.0282	9.0	61.0
0.1593	4.90	0.01259	2.0	68.0
0	5.40	-	-	70.0

Base (OH^-) added moles/$L.10^3$	pH	Free OH^- moles/ $L.10^3$	Bound OH^- moles/mole protein	Moles of H^+ dissociated per mole protein
1.1150	5.80	0.00001	14.0	84.0
1.6725	6.78	0.00006	21.0	91.0
2.2300	7.55	0.00040	28.0	98.0
3.3450	8.00	0.00100	42.0	112.0
5.5750	9.00	0.01070	70.0	140.0
6.1325	9.50	0.0340	76.0	146.0
8.3625	10.60	0.4260	99.0	169.0

(Fig. 5B)

TABLE 7.

Concentration of transfusion gelatin = 0.6% (0.8×10^{-4}M)

Concentration Zinc sulphate = 1.0×10^{-3}M

Ionic strength = 0.15 Temperature 25°C

H^+ added moles/ $L.10^3$	pH	Free H^+ moles/ $L.10^3$	Bound H^+ moles/mole protein	Moles of H^+ dissociated per mole protein
12.7450	2.20	7.1610	70.0	0
7.9660	2.61	2.7350	65.0	5.0
6.3730	3.00	1.1140	66.0	4.0
5.5760	3.27	0.5370	63.0	7.0
3.9830	3.72	0.1905	47.0	23.0
2.3898	4.28	0.0525	29.0	41.0
0.7966	4.90	0.0129	9.8	60.2
0.1593	5.00	0.0010	1.9	68.1
0	6.10	-	-	70.0

Base(OH^-) added moles/ $L.10^3$	pH	Free OH^- moles/ $L.10^3$	Bound OH^- moles/mole protein	Moles of H^+ dissociated per mole protein
1.1150	6.90	0.00008	13.9	83.9
2.2300	7.80	0.00063	27.8	97.8
3.3450	8.20	0.00160	41.8	111.8
5.5750	9.30	0.02010	69.0	139.0
6.1325	10.00	0.1000	75.4	145.4
8.3625	10.98	0.9627	92.5	162.5

(Fig. 5 C)

TABLE 8.

Concentration of transfusion gelatin = 0.6% (0.8×10^{-4}M)

Concentration of Cadmium sulphate = 0.88×10^{-3}M

Ionic strength = 0.15 Temperature 25°C

H^+ added moles/ $L.10^3$	pH	Free H^+ moles/ $L.10^3$	Bound H^+ moles/mole protein	Moles of H^+ dissociated per mole protein
12.7450	2.20	7.1610	70.0	0
7.9660	2.61	2.7350	65.0	5.0
6.3730	2.98	1.1670	65.0	5.0
5.5760	3.30	0.5010	63.5	6.5
3.9830	3.78	0.1660	47.7	22.3
2.3898	4.30	0.0501	29.2	40.8
0.7966	4.92	0.0120	9.8	60.2
0.1593	5.10	0.0079	1.8	68.2
0	6.42	-	-	70.0

Base (OH^-) added moles/ $L.10^3$	pH	Free OH^- moles/ $L.10^3$	Bound OH^- moles/mole protein	Moles of H^+ dissociated per mole protein
1.1150	7.2	0.00016	13.9	83.9
2.2300	8.0	0.00100	27.8	97.8
3.2450	8.5	0.00320	41.7	111.7
5.5750	9.2	0.01600	69.5	139.5
6.1325	9.9	0.08010	75.5	145.6
8.3625	10.5	0.32000	100.0	170.0

(Fig. 6A)

TABLE 9.

Concentration of transfusion gelatin = 0.6% (0.8×10^{-4}M)

Concentration of Cobalt Chloride = 1.0×10^{-3}M

Ionic strength = 0.15 Temperature 25°C

H^+ added moles/ $L.10^3$	pH	Free H^+ moles/ $L.10^3$	Bound H^+ moles/mole protein	Moles of H^+ dissociated per mole protein
12.7450	2.20	7.1610	70.0	0
7.9660	2.60	2.7990	65.0	5.0
6.3730	2.98	1.1670	65.0	5.0
5.5760	3.30	0.6010	63.5	6.5
3.9830	3.70	0.1995	47.0	23.0
2.3898	4.25	0.0562	29.0	41.0
0.7966	4.85	0.0141	9.7	60.3
0.1593	5.00	0.0010	1.9	68.1
0	6.48	-	-	70.0

Base (OH^-) added moles/ $L.10^3$	pH	Free OH^- moles/ $L.10^3$	Bound OH^- moles/mole protein	Moles of H^+ dissociated per mole protein
1.1150	7.38	0.00024	13.9	83.9
2.2300	8.05	0.00110	27.8	97.8
3.3450	8.25	0.00180	41.7	111.7
5.5750	9.40	0.02530	69.3	139.3
6.1325	9.90	0.08010	75.6	145.6
8.3625	10.55	0.35770	100.0	170.0

(Fig. 6 B)

TABLE 10.

Concentration of transfusion gelatin = 0.6% (0.8×10^{-4} M)

Concentration of nickel chloride = 1.008×10^{-3} M

Ionic strength = 0.15 Temperature 25°C

H^+ added moles/ $L.10^3$	pH	Free H^+ moles/ $L.10^3$	Bound H^+ moles/mole protein	Moles of H^+ dissociated per mole protein
12.7450	2.2	7.1610	70.0	0
7.9660	2.60	2.7990	65.0	5.0
6.3730	3.00	1.1140	66.0	4.0
5.5760	3.32	0.4786	63.7	6.3
3.9830	3.75	0.1778	47.5	22.5
2.3898	4.28	0.0525	29.2	40.6
0.7966	4.90	0.0128	9.8	60.2
0.1593	5.00	0.0010	1.9	68.1
0	6.45	-	-	70.0

Base (OH^-) added mole/ $L.10^3$	pH	Free OH^- moles/ $L.10^3$	Bound OH^- moles/mole protein	Moles of H^+ dissociated per mole protein
1.1150	7.12	0.00013	13.9	83.9
2.2300	8.10	0.00130	27.8	97.8
3.3450	8.65	0.00450	41.7	111.7
5.5750	9.40	0.02530	69.3	139.3
6.1325	10.02	0.10550	75.3	145.3
8.3625	10.88	0.76470	94.9	164.9

(Fig. 6 C)

TABLE 11.

Concentration of transfusion gelatin = 0.6% (0.8×10^{-4}M)

Ionic strength = 0.15 Temperature 30°C

H^+ added moles/ $L.10^3$	pH	Free H^+ moles/ $L.10^3$	Bound H^+ moles/mole protein	Moles of H^+ dissociated per mole protein
12.2934	2.23	6.6830	70.0	0
7.9029	2.70	2.2240	71.0	0
6.1467	3.15	0.7029	68.0	2.0
5.2686	3.50	0.3162	62.0	8.0
3.5724	4.00	0.1000	43.0	27.0
1.7562	4.60	0.0251	22.0	48.0
0.8781	4.90	0.0125	11.0	59.0
0	5.56	-	-	70.0

(Fig. 7A)

Concentration of transfusion gelatin = 0.6% (0.8×10^{-4}M)

Concentration of lead nitrate = 0.4×10^{-3}M

Ionic strength = 0.15 Temperature 30°C

H^- added moles/ $L.10^3$	pH	Free H^- moles/ $L.10^3$	Bound H^- moles/mole protein	Moles of H^+ dissociated per mole protein
12.2934	2.22	6.8370	68.0	2.0
7.9029	2.65	2.4940	67.0	3.0
6.1467	3.08	0.8709	66.0	4.0
5.2686	3.30	0.5011	59.5	9.5
3.5124	3.70	0.1995	41.0	29.0
1.7562	4.30	0.0501	21.0	49.0
0.7025	4.70	0.0199	9.0	61.0
0	5.58	-	-	-

(Fig. 7B)

A pH titration curve may be split into three portions; (i) in the region pH3 to pH5 where carboxyl groups are deprotonated; (ii) the neutral pH region ($\approx$ between 6 to 7) where imidazole groups are available and (iii) alkaline side of pH, where groups other than carboxyl and imidazole are freely available for the binding of metal ions.

The experimental results show that the activity of hydrogen ions is greatly increased in presence of metal ions. It was, therefore, postulated that the large increase in activity of hydrogen ions might be due to competition between the metal and the hydrogen ions for a common site (by competition is meant that a metal and hydrogen ion cannot occupy any particular single site at the same time). If ions of both species are in equilibrium with a particular site on the protein molecule, the number of either species bound to these sites depends upon their respective affinities and activities. In view of the above and with the available detailed information about the hydrogen ion equilibria[6] of transfusion gelatin the following inferences may be drawn.

(i) The interaction of metal ions at pH 3-5 is a competitive one; where metal ion competes with the hydrogen ions for carboxyl groups. Hence a metal ion under consideration, if combines with the carboxyl groups, the

activity of hydrogen ions should increase in presence of metal ion at this pH. On the other hand, at pH 6 and above, the carboxyl groups are in the basic (deprotonated) form, therefore, the reaction is non competitive one, which means the metal ion may combine with any number of fully deprotonated carboxyl groups available at this pH. Thus there would be no substantial effect on hydrogen ion equilibria, if a metal ion is introduced in the system.

(ii) At pH 6.5, if a metal combines with the imidazole groups, it has to compete with the hydrogen ion. In doing so, metal ions replace hydrogen ions, resulting in a decrease in pH and consequently the hydrogen ion equilibria will be shifted in the direction of the basic side of the functional groups.

Copper-transfusion gelatin system

Concentration of cupric chloride = 2.0×10^{-3}M

Concentration of transfusion gelatin = 0.8×10^{-4}M

pH	H^+ dissociated in presence of metal ions	H^+ dissociated in absence of metal ions.	Difference or V_M	Free metal ions at equilibrium x 10^{-3}M	log K
3.0	7	4	3	-	-
4.0	39	30	9	-	-
5.0	70	60	10	-	-
5.5	79	69	10	1.2	2.05
6.0	86	74	12	-	-
7.0	94	82	12	1.04	3.28
8.0	113	93	20	-	-
9.0	140	120	20	0.40	3.04

The titration curve of copper-transfusion gelatin mixture differs markedly from that of the transfusion gelatin alone. The activity of hydrogen ion is greatly increased when copper is introduced in the system, and the hydrogen ion equilibria has been shifted in the direction of the basic side of the functional groups (Fig.5B). The only explanation which could be offered for such a behaviour is, that the cupric ion combines with the carboxyl groups and very well compete with the hydrogen ion in the pH range 3 - 5. At higher pH (5 - 7), another reactive site i.e., imidazole groups, comes into picture. Contrary to the behaviour of copper-transfusion gelatin complex at dropping mercury electrode, the pH-metric results show that copper combines with the imidazole groups. The increased activity of hydrogen ions in the pH range 5 to 7 cannot be attributed to the combination either with the carboxyl groups or amino groups, since metal-carboxyl group reaction is essentially a non-competitive one, and amino groups are in the acid from in this pH range. The only logical conclusion, therefore, is that the copper enters into combination with the imidazole groups, replacing hydrogen ions. Tanford concluded, on the basis of polarographic measurements, that cupric ions got bound only to the imidazole groups of serum albumin, whereas Rao and Lal arrived at the conclusion that in binding cupric ions, imidazole groups are involved, followed by an increasing participation of carboxyl groups; a fact which

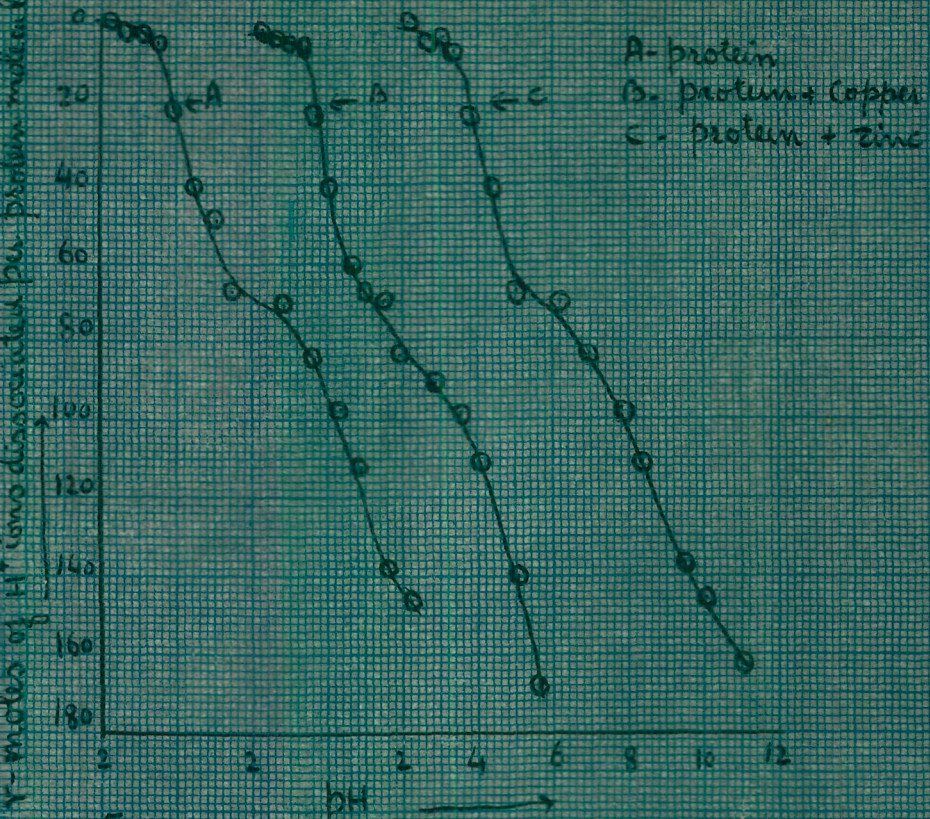

A- protein
B- protein + Copper
C- protein + Zinc
γ-moles of H+ ions dissociated per protein molecule
pH

Fig. 5

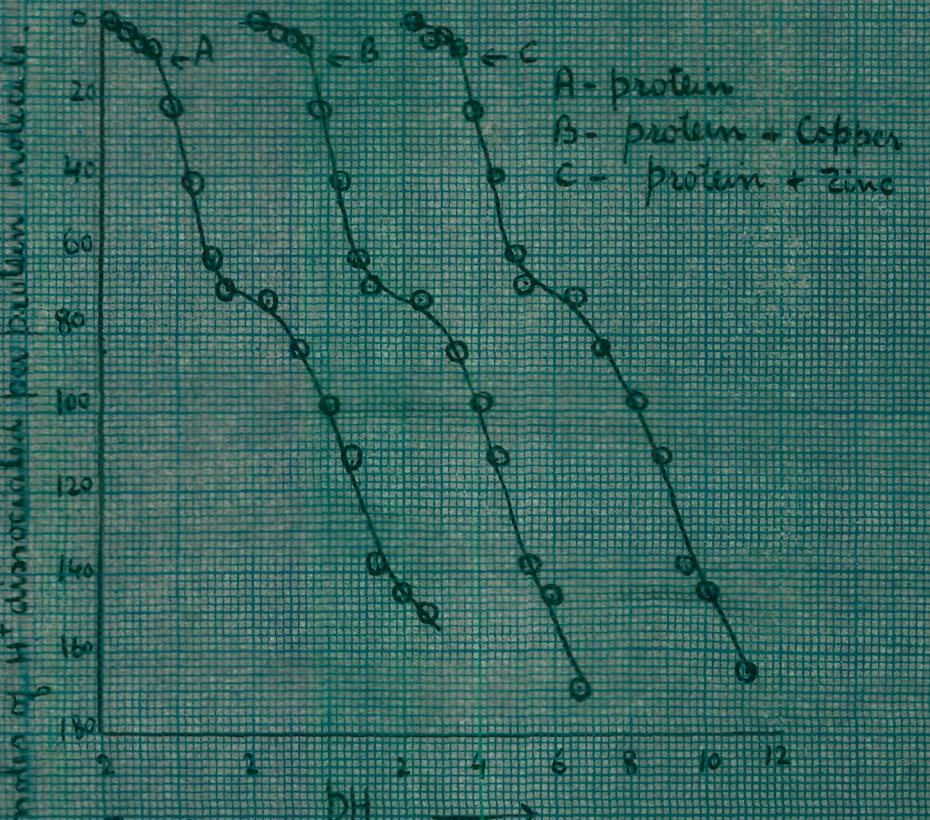
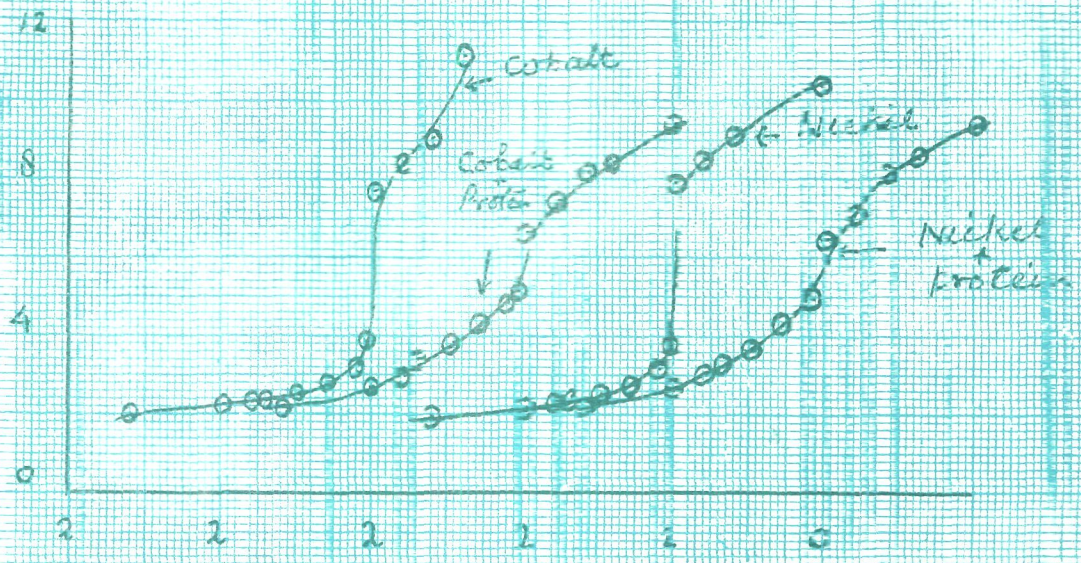

A- protein
B- protein + Copper
C- protein + Zinc
γ-moles of H+ ions dissociated per protein molecule
pH

Fig 6



Volume of acid or alkali added in ml →

Fig-3

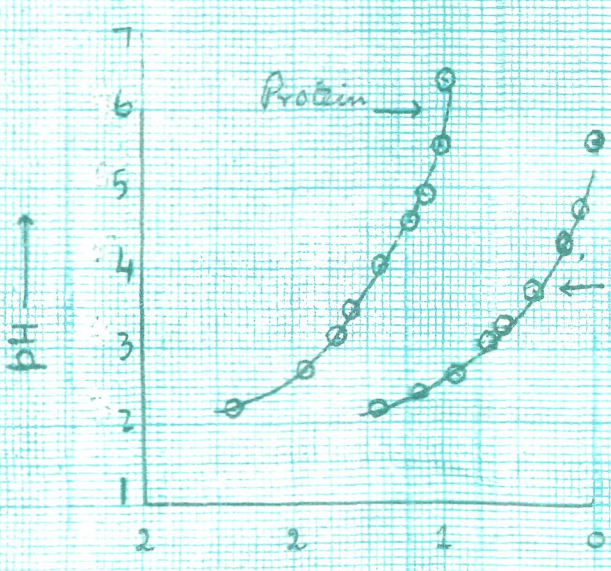


Fig-4

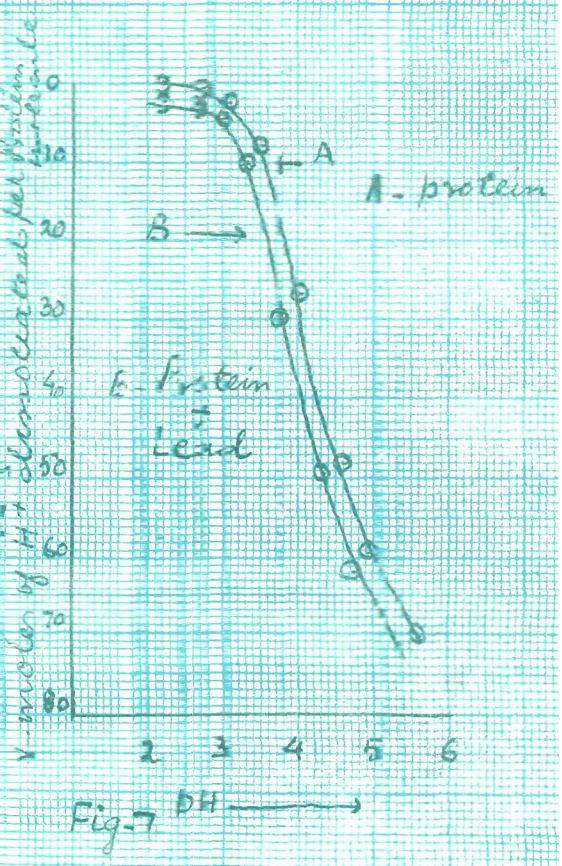


Fig-7

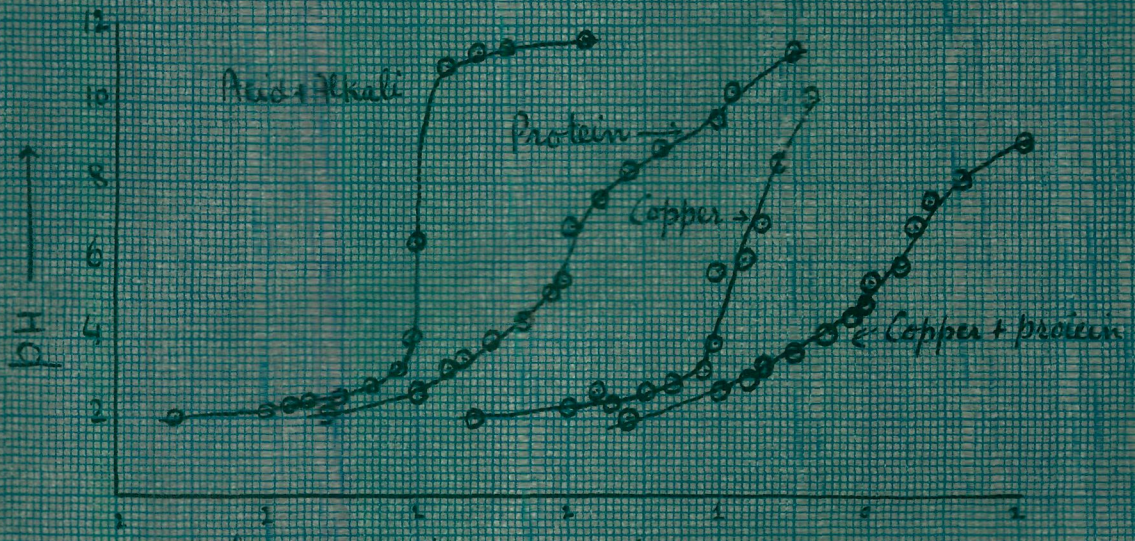

12
10
8
6
4
2
pH
Acid + Alkali
Protein
Copper
Copper + protein
Volume of acid or alkali added (m.l)
Each curve starts at 2 m.l of acid

Fig. 1

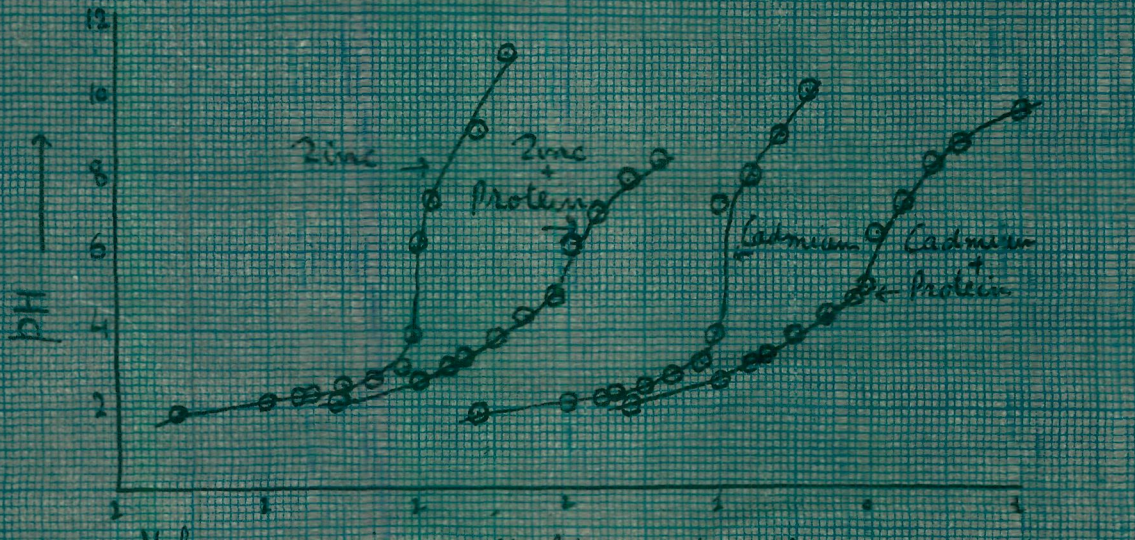

12
10
8
6
4
2
pH
Zinc
Zinc + Protein
Cadmium
Cadmium + Protein
Volume of acid or alkali added (m.l)
Each curve starts at 2 m.l of acid.

Fig. 2

is realised by the present pH-metric studies in case of fibrous protein. Further confirmation is forthcoming on the basis of the results given under equilibrium dialysis studies (Chapter 3).

The values of V_M as calculated from the titration curves come out to be 10 and12 at pH 5.5 and 7 respectively, for the total metal concentration of 2.0×10^{-3}M. The intrinsic association constants, for the combination of Cu-carboxyl groups and Cu-imidazole groups of transfusion gelatin are as follows:

$$\log K_{COO} = 2.02$$

$$\log K_{im.} = 3.28$$

and free energy change come to be -2.743 KCal and -4.452 Kcal. respectively.

Interaction of Zn, Cd, Co, Ni and Pb with transfusion gelatin.

Concentration of $ZnSO_4 = 1 \times 10^{-3}$M

Concentration of transfusion gelatin = 0.8×10^{-4}M

pH	H^+ dissociated in presence of metal ions	H^+ dissociated in absence of metal ions	Difference or V_M	Free metal ions at equilibrium $\times 10^{-3}$M	Log K
3	5	4	1	-	-
4	32	30	2	-	-
5	64	60	4	-	-
5.5	73	69	4	0.68	1.87
6.0	79	74	5	-	-
7.0	87	82	5	0.60	2.74
8.0	102	93	9	-	-
9.0	120	120	9	0.28	2.87

Concentration of $CdSO_4$ = $0.88 \times 10^{-3}M$

Concentration of transfusion gelatin = $0.6 \times 10^{-4}M$

pH	H^+ dissociated in presence of metal ions	H^+ dissociated in absence of metal ions	difference or V_M	Free metal ions at equilibrium $\times 10^{-3}M$	log K
3.0	4.0	4	0	-	-
4.0	32.0	30	2	-	-
5.0	63.0	60	3	-	-
5.5	72.0	69	3	0.64	1.76
6.0	77.0	74	3	-	-
7.0	84.0	82	2	-	-
8.0	99.0	93	6	-	-
9.0	127.0	120	7	0.23	-

Concentration of $CoCl_2$ = $1.0 \times 10^{-3}M$

Concentration of transfusion gelatin = $0.8 \times 10^{-4}M$

3.0	5	4	1	-	-
4.0	34	30	4	-	-
5.0	65	60	5	-	-
5.5	74	69	5	0.6	2.02
6.0	78	74	4	-	-
8.0	99	93	6	-	-
9.0	126	120	6	0.52	-

Concentration of $NiCl_2$ = 1.008×10^{-3}M

Concentration of transfusion gelatin = 0.8×10^{-4}M

pH	H^+ dissociated in presence of metal ions	H^+ dissociated in absence of metal ions	difference or V_M	Free metal ions at equilibria $\times 10^{-3}$M	log K
3.0	4.0	4.0	0	-	-
4.0	32.0	30.0	2	-	-
5.0	64.0	60.0	4	-	-
5.5	73.0	69.0	4	0.688	1.86
6.0	78.0	74.0	4	-	-
7.0	85.0	82.0	3	-	-
8.0	98.0	93.0	5	-	-
9.0	126.0	120.0	6	-	-

Concentration of $Pb(NO_3)_2$ = 0.4×10^{-3}M

Concentration of transfusion gelatin = 0.8×10^{-4}M

2.0	2.0	0	2	-	-
3.0	4.0	2.0	2	-	-
4.0	36.0	33.0	3	-	-
5.0	64.0	62.0	2	-	-
5.5	72.0	70.0	2	0.24	2.007
6.0	-	-	-	-	-

A comparison of the experimental data reveals the following interesting features of the interaction process.

(i) Zinc and cadmium both appear to combine with the carboxyl groups of transfusion gelatin. A some what greater shift in hydrogen ion equilibria, in the direction of basic side of the functional groups, is observed in presence of zinc than with the cadmium transfusion gelatin system, showing thereby that zinc-carboxyl type binding is stronger than the cadmium-carboxyl binding. A marked difference between the two systems is observed in the neutral pH range, where zinc shows a definite binding with the imidazole groups and on the other hand cadmium appears to have a very little affinity for imidazole groups.

(ii) The pH-metric results for the systems cobalt-transfusion gelatin, nickel-transfusion gelatin and lead-transfusion gelatin are characterised by the binding of the metal ions at the carboxyl groups of the protein. The log K values for the above system calculated assuming one to one binding come out to be 2.02, 1.86 and 2.007 respectively. The participation of carboxyl groups in the interaction process appears to increase in the direction

$$Cd < Ni < Zn < pb < Co$$

(iii) The hydrogen ion equilibria of transfusion gelatin is not influenced by these metal ions except zinc in the neutral pH region. It may, therefore, be calculated that except zinc none of these metal ions combine with the imidazole groups.

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(c) CHAPTER 3.

Equilibrium dialysis studies on the interaction of Copper, Zinc and Cadmium with transfusion gelatin.

I N T R O D U C T I O N

In the preceding two chapters, the results of the studies on metal transfusion gelatin interaction have been described, employing the polarographic and pH-metric techniques. Lately another method, namely equilibrium dialysis has come into vogue and is finding increasing application in such studies. The method has some obvious advantage over the other methods[1], due to the following reasons: (i) it provides more accurate data on the binding of metal ions, (ii) the binding data may be measured directly; with the results that one has not to seek the solution of the problem from the indirect data, on the extent of complex formation, (iii) less elaborate equipment is required than for most of the other procedures.

Early reference available in the chemical literature is that of Obsrne[2], who pointed out the great utility of the dialysis technique, as a valuable tool for the study of protein interaction. But the work did not get enough recognition until J.H. Northrop and M. Kunitz[3-5] have extensively employed the technique for the quantitative study of metal protein complexes. Equilibrium dialysis method was put to more productive use by Klotz and Curme[6]. Since then a number of papers have appeared

on this aspect, the important contributors being; Klotz and Fiss[7-8], Klotz and Co-workers (binding of Cu(ii) to α-Casein, β-Lactoglobulin, γ-globulin and Lysozyme); Gurd and Goodman[11-12] (binding of Zn(ii) to human serum albumin in the pH range 5 - 68); Sarff and Mark[13] (interaction of Zn(ii) with bovine serum albumin); Gurd[14] (binding of Cd to the imidazole groups of human serum albumin); Cunningham et al[15] (binding of Zn(ii) to insulin); Gurd and Murray[16] (complex formation between Pb and human serum albumin); Katz and Klotz[17] (binding of Ca to bovine serum albumin), Rao and Lal[18] (the combination of Cu, Zn and Cd to bovine serum albumin) and Breslow and Gurd[19] (interaction of cupric and zinc ions with sperm whale Metmyoglobin).

From the foregoing review it is evident, that although enough work has been done to investigate the nature of metal complexes of simple globular proteins, such as serum albumin; much remains to be done to elucidate the complexes of fibrous protein like gelatin by the method of equilibrium dialysis. To achieve this, investigations on the binding of copper, zinc and cadmium to the transfusion gelatin were undertaken. The results of these studies are described in this chapter. While those of pH-metric investigations have already been fully described and discussed in chapter 2.

EXPERIMENTAL

The equilibrium dialysis technique consists of a vessel divided into two compartments by a membrane usually prepared from cellulose sausage casings, which is permeable to water and small ions but completely impermeable to protein molecule[1]. If the protein is confined to one compartment and if it binds metal ions, then at equilibrium the total metal concentration in the protein compartment is greater than that in the outside one (protein free). The difference in the two concentrations is a measure of the metal ion bound to the protein. The solutions in the two compartments are analysed for the metal content by standard analytical or spectrophotometric methods.

In practice two possible sources of error may creep in the dialysis technique; (i) some asymmetry in the distribution of metal ions may exist because of the Donnan effect[20]; and so the allowance should be made for this in the calculation[21], or an inert electrolyte may be added to eleminate it; (ii) some adsorption of metal ions may occur on the membrane itself. In the latter case suitable correction can be made by setting up controlled dialysis, in which the protein is absent and then measuring the depletion of the metal ions from the solution by usual methods.

of supporting electrolyte to be used in the binding measurements.

Filling the dialysis bags

A piece of dialysis casing was removed from the test solution in which it was soaked, and the excess liquid was removed carefully. A square knot was tied in one end of the casing; 5 m.l. of protein solution was pipetted in. Most of the air was forced out, and the bag was closed with two overhead knots, one on top of the other. The upper knot was a bit away from the lower one, making a loop, than the bag was suspended by means of thread holding it with the loop, in 5 m.l. protein free metal solution[22].

Solutions and Reagents

Transfusion gelatin (6 % concentration, Molecular weight, 75,000) was used throughout these investigations.

A stock solution of the protein in the supporting electrolyte (0.15M) was prepared, and pH was adjusted to the desired value.

Chemically pure (E. Merck) samples of cupric chloride, zinc sulphate, cadmium sulphate were dissolved

Apparatus and Technique

The apparatus consisted of a machnical shaker, provided with holes for equilibriation tubes. Pyrex test tubes closed by means of rubber stoppers (the rubber stoppers are covered with thin sheets of polysterine) were used as dialysis vessels. Adequate shaking was effected by keeping in the shaker in a water thermostate (townson and Mercer Co. Croydon) all measurements were made at 25 $\pm$ 0.1°C.

pH measurements were carried out by means of Backman Model G. pH meter, using glass electrode.

Preparation of Dialysis bags

The visking sausage casing (23/32 inch. in diameter) supplied by the Director National Chemical Laboratories, Poona, India, was used as the dialysis bags. 15 to 16 cm lengths of visking sausage casings were cut from main strip and put into a beaker of distilled water and heated on a steam bath for an hour. Heating was repeatedly done with fresh distilled water. The casings were next soaked at room temperature for a few hours. This procedure was repeated several times until the final washings were free from sulphur. The casings were then soaked for about 6 hours in a solution

in triply distilled water (distilled in all glass apparatus)[22] to get their respective solutions. Metal content of the stock solutions were determined as described earlier by complexometric titration[23]. A.R. reagent grade potassium chloride and potassium hydroxide were used to prepare their solution, these solutions were used to maintain the constant ionic strength and pH, respectively. Solutions of EDTA (E. Merck), E. Black T and Murexide were prepared as recommended by Schwarzenbach[23].

Procedure

(i) Varying amounts of cupric chloride (7.795×10^{-3} M) viz., 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 m.l. were mixed in different Pyrex glass test tubes, with requisite amount of potassium chloride and the total volume made upto 10 m.l. with the ionic strength being kept at 0.15. 5 m.l. of each of the solution was pipetted in a clean and dry dialysis tube. A dialysis bag containing 5 m.l. protein solution (2.4 %) of pH 5.5 was suspended in each tube carefully, so that the thread should not come in contact with the solution. The remaining 5 m.l. of each of the solutions were used for blank experiment under identical set of conditions; in which the dialysis bag contained 5 m.l. protein-free supporting electrolyte solution of the same pH.

A similar set of solutions were arranged, containing the same metal concentration, with the only difference that the dialysis bags contained 5 m.l. protein solution (2.0%) of pH 7.5.

(ii) Varying amounts of zinc sulphate (4.408×10^{-3}M) viz., 0.48, 1.0, 2.0, 3.0, 4.0, 5.0, 6.2, 7.2, 8.2 and 9.0 m.l. were mixed with requisite amount of potassium chloride, in different pyrex test tubes and total volume made upto 10 m.l. with the ionic strength being kept at 0.15. 5.0 m.l. of each of the solution was pipetted in a clean and dry dialysis tubes. A dialysis bag containing 5 m.l. of protein solution (2.4%) of pH 5.5 was suspended in each tube carefully so that the thread should not come in contact with the solution, 5 m.l. remaining solution was used for blank experiment under identical set of conditions, in which the dialysis bag contained 5 m.l. of protein free supporting electrolyte solution of same pH.

Similar set of solutions were arranged, containing the same metal concentration, with the only difference that the dialysis bags contained 5 m.l. of protein solution (2.0%) of pH 7.5.

(iii) Varying amounts of cadmium sulphate (4.21×10^{-3}M) viz., 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 m.l. were mixed in different test tubes with requisite

amount of potassium chloride so as to maintain the ionic strength at 0.15. Total volume was made upto 10 m.l., 5 m.l. of each of the solution was equilibriated against 5 m.l. of protein solution (2.4%) of pH 5.5. Another 5 m.l. of each of the solution was used for blank.

Similar set of solutions were arranged, containing the same metal concentration, with the only difference that the dialysis bags contained 5 m.l. of protein solution (2.0%) of pH 7.5.

The dialysis tubes were placed on the shaker, and shaked machnically for the required period (as determined previously). The time to attain equilibrium was found to be 30, 38 and 40 hours for copper, zinc and cadmium respectively. After the required period, the dialysis bags were withdrown from the tubes. An accurately measured volumes of the external solution was analysed for metal ions. The blank tubes were employed to determine the amount of metal ions bound to the material of dialysis bag. These amounts for Cu, Zn and Cd were found to be negligibily small.

TABLE 1.

Concentration of transfusion gelatin = 2.4 %

Total volume inside the bag = 5 m.l.

Total volume outside the bag = 5 m.l.

Total ionic strength inside and outside = 0.15

Temperature 25 ± 0.1°C

Time of equilibriation 30 hours

pH of the protein solution = 5.5

Initial concentration of metal ions x 10^{-3}M ($CuCl_2$)	Concentration of metal ions after equilibration	pH of the protein solution
0.3898	0.0698	5.48
0.7795	0.1715	5.45
1.1693	0.2733	5.44
1.5590	0.3430	5.42
1.9488	0.3808	5.40
2.3385	0.4185	5.37
2.7283	0.4873	5.35
3.1180	0.5900	5.32
3.5078	0.6918	5.30
3.8975	0.7775	5.30
4.2873	0.9273	5.30
4.6770	1.1570	5.30

TABLE 2.

Concentration of transfusion gelatin = 2.0 %

Total volume inside the bag = 5 m.l.

Total volume outside the bag = 5.m.l.

Total ionic strength inside and outside = 0.15

Temperature 25 ± 0.1°C

Time of equilibration 30 hours

pH of the protein solution = 7.5

Initial concentration of metal ions x 10^{-3}M ($CuCl_2$)	concentration of metal ions after equilibration	pH of the protein solution
0.3898	0.0431	7.4
0.7795	0.1128	7.35
1.1693	0.2092	7.30
1.5590	0.2789	7.30
1.9488	0.3220	7.28
2.3385	0.3650	7.26
2.7283	0.4347	7.24
3.1180	0.5311	7.20
3.5078	0.6275	7.10
3.8975	0.7611	7.00
4.2873	=	=
4.6770	=	=

TABLE 3.

Concentration of transfusion gelatin = 2.4 %

Total volume inside the bag = 5 m.l.

Total volume outside the bag = 5 m.l.

Total ionic strength inside and outside = 0.15

Temperature ± 25 0.1°C

Time of equilibration 38 hours

pH of the protein solution = 5.5

Initial concentration of metal ions x 10^{-3}M($ZnSo_4$)	Concentration of metal ions after equilibration x 10^{-3}M	pH of the protein solution
0.2318	0.0718	5.49
0.4830	0.1630	5.48
0.9660	0.3270	5.46
1.4490	0.4870	5.45
1.9320	0.6490	5.45
2.4150	0.8150	5.44
2.9946	1.0746	5.42
3.4776	1.2356	5.40
3.9606	1.3966	5.40
4.1470	1.5040	5.40

TABLE 4.

Concentration of transfusion gelatin = 2.0 %

Total volume inside the bag = 5 m.l.

Total volume outside the bag = 5 m.l.

Total ionic strength inside and outside = 0.15

Temperature 25 ± 0.1°C

Time of equilibration 38 hours

pH of the protein solution = 7.5

Initial concentration of metal ions x 10^{-3}M ($ZnSc_4$)	Concentration of metal ions after equilibration x 10^{-3}M	pH of the protein solution
0.2318	0.0985	7.45
0.4830	0.2263	7.40
0.9660	0.2993	7.25
1.4490	0.4355	7.20
1.9320	0.5985	7.15
2.4150	0.7615	7.10
2.9946	1.0477	7.10
3.4776	1.2373	7.05
3.9606	1.3737	7.00
4.1470	1.4800	7.00

TABLE 5.

Concentration of transfusion gelatin = 2.4 %

Total volume inside the bag = 5 m.l.

Total volume outside the bag = 5 m.l.

Total ionic strength inside and outside = 0.15

Temperature 25 ± 0.1°C

Time of equilibration 40 hours

pH of the protein solution = 5.5

Initial concentration of metal ions x 10^{-3}M ($CdSo_4$)	Concentration of metal ions after equilibration x 10^{-3}M	pH of the protein solution
0.2105	0.0515	5.49
0.4210	0.1753	5.48
0.8420	0.2660	5.48
1.2630	0.3830	5.45
1.6840	0.4360	5.45
2.1050	0.6330	5.44
2.5260	0.7140	5.42
2.9470	0.8510	5.40
3.3680	0.9360	5.40
3.7890	1.2930	5.40

TABLE 6.

Concentration of transfusion gelatin = 2.0 %

Total volume inside the bag = 5 m.l.

Total volume outside the bag = 5 m.l.

Total ionic strength inside and outside = 0.15

Temperature 25 ± 0.1°C

Time of equilibration 40 hours

pH of the protein solution = 7.5

Initial concentration of metal ions x 10^{-3}M($CdSO_4$)	Concentration of metal ions after equilibration x 10^{-3}M	pH of the protein solution
0.2105	0.0805	7.48
0.4210	0.1543	7.47
0.8420	0.3219	7.46
1.263	0.5029	7.45
1.6840	0.6039	7.38
2.1050	0.8382	7.35
2.5260	0.9258	7.35
2.9430	1.1334	7.33
3.3680	1.2611	7.32
3.7890	1.6554	7.32

RESULT AND DISCUSSION

The experimentally measured quantity which is used in the evaluation of the stoichiometry and intrinsic association constant of metal-protein complexes; is the number of moles of metal bound per mole of protein (or per specific weight of the protein) which is given by the expression.

$$V_M = \frac{M_P}{P_T}$$

where M_P is the number of moles of protein bound metal, in a dialysis tube and P_T, is the total number of moles of protein per tube. If the dialysing membrane does not bind any of the ligand (metal or protein), and if the Donnan effect is negligible, the calculation of V_M would be extremely simple. The value of M_P is found from the following relationship.

$$M_P = M_T - V_T\ (M)$$

where M_T is the total amount of metal ions in the tube, V_T, is the total volume of the solution, and (M) is the concentration of metal ions in the protein free external solution. Actually most ligand are bound by the dialysing bags, so the above equation is modified in the form

$$M_P = M_T - V_T\ (M) - MB$$

where M_B is the number of moles of metal bound to the bag. Three methods have been used for the determination of M_B. The simplest of these is the direct comparison method. It yields approximate results which are nearly correct

only when the bag binding is sufficiently small.

Direct Comparison Method.[1]

A blank tube containing the same amount of each non protein component is equilibrated, and from the know - ledge of the difference in concentration of metal inside and outside the bag, the amount of metal bound to the bag (M_B) is calculated as

$$M_B = M_T - V_T (M)'$$

where $(M)'$ is the concentration of unbound metal in the blank tube. Since the bag binding is sufficiently small (in the range 0.5×10^{-5} to 1×10^{-6} M in the present case), direct comparison method has been utilised in the present studies.

The binding data have been represented graphically for the metal protein complex formation, as a function of free metal concentration on a semilogarithm graph (extent of binding is plotted against the logarithm of the concentration of unbound metal ions). The results are summarised in the following tables.

The intrinsic association constants for the system, Cu-transfusion gelatin, Zn-transfusion gelatin and Cd-transfusion gelatin are calculated applying Scatchard equation.[24]

TABLE 7.

The Equilibrium between Cu^{++} and transfusion Gelatin At pH 5.5.

Concentration of transfusion gelatin = 3.2×10^{-4} M

Ionic strength = 0.15 Temperature 25°C

pH	Bound metal ions x 10^{-3} M	Free metal ions x 10^{-3} M	V_M	log K
5.48	0.320	0.0698	1.0	2.237
5.45	0.608	0.1715	1.9	2.130
5.44	0.896	0.2733	2.8	2.100
5.42	1.216	0.3430	3.8	2.140
5.40	1.568	0.3808	4.9	2.211
5.37	1.920	0.4185	6.0	2.264
5.35	2.241	0.4873	7.0	2.270
5.32	2.528	0.5900	7.9	2.245
5.30	2.816	0.6918	8.8	2.033
5.30	3.120	0.7775	9.75	2.227
5.30	3.360	0.9273	10.5	2.187
5.30	3.520	1.1570	11.0	2.115

(Fig. 1 A)

N.B. The average value of log K comes out to be = 2.179

∴ and the value free energy change of the combination

$-\Delta F = 2.972$ KCal.

TABLE 8.

The Equilibrium between Cu^{++} and Transfusion gelatin at pH 7.5

Concentration of transfusion gelatin = 2.667×10^{-4}M.

Ionic strength = 0.15 Temperature 25°C.

pH	Bound metal ions x 10^{-3}M	Free metal ion x 10^{-3}M	V_M	log K
7.4	0.3467	0.0431	1.3	-
7.35	0.6667	0.1128	2.5	3.345
7.30	0.9601	0.2092	3.6	3.240
7.30	1.2801	0.2789	4.8	3.253
7.28	1.6268	0.3220	6.1	3.316
7.26	1.9735	0.3650	7.4	3.379
7.24	2.2936	0.4347	8.6	3.419
7.20	2.5869	0.5311	9.7	3.426
7.10	2.8803	0.6275	10.8	3.502
7.00	3.1364	0.7611	11.76	3.420

(Fig. 1 B)

NB. The average value of log K comes out to be = 3.366 and the value free energy change of the combination $-\Delta F = 4.589$ KCal

TABLE 9.

The Equilibrium between Zn^{++} and Transfusion gelatin at pH 5.5

Concentration of transfusion gelatin = 3.2×10^{-4}M

Ionic strength = 0.15 Temperature 25°C

pH	Bound metal ions x 10^{-3}M	Free metal ions x 10^{-3}M at equilibrium	V_M	log K
5.49	0.160	0.0718	0.5	1.921
5.48	0.320	0.1630	1.0	1.879
5.46	0.639	0.3270	1.9	1.850
5.45	0.692	0.4870	3.0	1.880
5.45	1.283	0.6490	4.0	1.886
5.44	1.600	0.8150	5.0	1.890
5.42	1.920	1.0746	6.0	1.855
5.40	2.242	1.2356	7.0	1.866
5.40	2.564	1.3966	8.0	1.877
5.40	2.643	1.5040	8.3	1.862

(Fig. 2 A)

N.B. The average value of log K comes out to be = 1.876 and the value free energy change of the combination i.e. $-\Delta F$ = 2.558 KCal

TABLE 10.

The Equilibrium between Zn^{++} and Transfusion gelatin at pH 7.5

Concentration of transfusion gelatin = 2.667×10^{-4} M

Ionic strength = 0.15 Temperature 25^{o}C

pH	Bound metal ions $\times 10^{-3}$ M	Free metal ions $\times 10^{-3}$ M at equilibrium	V_M	log K
7.45	0.1333	0.0985	0.5	-
7.40	0.2667	0.2263	1.0	-
7.25	0.6667	0.2993	2.5	2.922
7.20	1.0135	0.4355	3.8	2.912
7.15	1.3335	0.5985	5.0	2.921
7.10	1.6535	0.7615	6.2	2.942
7.10	1.9469	1.0477	7.3	2.863
7.05	2.2403	1.2373	8.4	2.849
7.00	2.5869	1.3737	9.7	2.978
7.00	2.6670	1.4800	10.0	-

(Fig. 2 B)

N.B. The average value of log K comes out to be = 2.912 and the value free energy change of the combination $-\Delta F$ = 3.971 KCal

TABLE 11.

The Equilibrium between Cd^{++} and Transfusion gelatin at pH 5.5

Concentration of transfusion gelatin = 3.2×10^{-4}M

Ionic strength = 0.15 Temperature 25°C

pH	Bound metal ion x 10^{-3}M	Free metal ions x 10^{-3}M at equilibrium	V_M	log. K
5.49	0.159	0.0515	0.50	2.065
5.48	0.256	0.1753	0.80	1.739
5.46	0.576	0.2660	1.80	1.915
5.45	0.880	0.3830	2.75	1.946
5.45	1.248	0.4360	3.91	2.048
5.44	1.472	0.6330	4.60	1.961
5.42	1.812	0.7140	5.85	2.020
5.40	2.096	0.8510	6.55	1.997
5.40	2.432	0.9360	7.60	2.026
5.40	2.496	1.2930	7.80	1.898

(Fig. 3 A)

N.B. The average value of log K comes out to be = 1.962 and the value of free energy change of the combination $-\Delta F$ = 2.675 KCal

TABLE 12.

The Equilibrium between Cd^{++} and Transfusion gelatin at pH 7.5

Concentrations of transfusion gelatin = 2.667×10^{-4}M

Ionic strength = 0.15 Temperature = 25°C

pH	Bound metal ions x 10^{-3}M	Free metal ions x 10^{-3}M at equilibrium	V_M	log K
7.48	0.1390	0.0805	0.60	-
7.47	0.2667	0.1543	1.00	-
7.46	0.5201	0.3219	1.95	-
7.45	0.7601	0.5029	2.85	-
7.38	1.0801	0.6039	4.05	-
7.35	1.2668	0.8382	4.75	-
7.35	1.6002	0.9258	6.00	-
7.33	1.8136	1.1334	6.80	-
7.32	2.1069	1.2611	7.90	-
7.32	2.1336	1.6554	8.00	-

(Fig. 3 B)

The interpretation of the experimental results on the studies of the metal protein system, are fraught with difficulties due to the complex physico-chemical nature of the protein molecule[25]. Several general properties of the protein must be taken into account, before any attempt is made to arrive at a definite conclusion. First of all the length of the peptide chain is much greater than in most compounds (amino acids and peptides etc.) which are employed as a model for the comparison and interpretation of the data on metal-protein interaction (e.g. the affinity of a particular metal ion for acetate, imidazole and ammonia has been frequently compared to the carboxyl, imidazole and amino groups of the protein). Due to this long peptide chain; the side chain groups have got much more importance in binding metal ions than the terminal amino or carboxyl groups. Secondly, there are usually several numbers of a given class of groups in a protein molecule, with the result, that the binding of metal ions is dependent both on the intrinsic affinity and on the number of groups available for interaction. Thirdly, the ligand groups in a protein molecule are not free to move, therefore do not behave as a simple classical ligand. Finally the protein molecule normally bears a considerable number of positive and negative charges, whose net potential field may favour or hinder the approach of the metal ion. This means, that the apparent affinity constant changes as more and more ions are taken up or given off by the

protein molecule.

Apart from this, one has to consider the factors associated with the Chemistry of simple metal complexes[25], e.g. co-ordination number, hydration as well as the hydrolysis of metal ions, competition with hydrogen ions and chelate formation. Such data are summarised in the following table[25].

TABLE 13.

System	Z (Co-ordination number)	log K.	Approximate pH, onset of precipetation.
Zn-Imidazole	4	2.76[26]	6
Zn-Acetate	-	1.03	-
Cd-Imidazole	4	2.80[27]	7
Cd-Acetate	-	1.30	-
Cu-Imidazole	4	4.36	6
Cu-Acetate	-	2.16	-

Copper - transfusion gelatin system

In the preceeding chapter, it has been argued on the basis of pH-metric studies, that copper has got a strong affinity for imidazole groups of transfusion gelatin. Furthermore, carboxyl groups also participate in the interaction process. This state of affairs seems to be true since

data obtained by equilibrium dialysis technique points towards the same conclusion.

Copper-transfusion gelatin system, studied at pH 5.5 shows clearly the binding of metal ions at the carboxyl groups. As the concentration of metal ions is increased from 0.389×10^{-3}M to 0.4677×10^{-2}M, V_M rises progressively until a value of 10 or 11 is reached. These results indicate that the binding is not complete even in this large range of concentration, and no evidence of saturation limit being reached is forthcoming. On the other hand, V_M versus log Co (Co being the concentration of free metal ions at equilibrium) curve indicates a slight decrease in the uptake of metal ions by the protein molecule.

The constancy of log K values (Table 7, column 5) over a wide range of metal concentration, and a measurable depletion in the pH of the protein solution (pH of the protein solution was adjusted to 5.5, and after equilibration it decreased to 5.3) on keeping in the dialysis bag, suggest the binding of cupric ions to the carboxyl groups; and support the view that the competition exists between the hydrogen ion and cupric ion for the common site. The results on the intrinsic association constant (as calculated applying Scatchard equation[24], assuming the competition between cupric and hydrogen ion, and inserting the values of V_M) obtained by this method also fall within the comparable values of log K for the Cu-acetate complex.

With the increase in pH, the values of V_M show an increase (from 9.75 to 11.75 for a change of pH 5.3 to 7.0; for total metal concentration of 0.3897×10^{-2} M) pointing towards the fact that the imidazole groups of transfusion gelatin also participated in the binding process. Besides, a marked decrease in pH of the protein solution (from 7.5 to 7.0) is observed after equilibration. Such a decrease may be attributed to the replacement of hydrogen ions from the acidic groups of transfusion gelatin and the only possible way by which such a replacement can be affected is by the binding of cupric ion to the imidazole groups of the protein. Moreover the constancy of the log K values over a wide concentration range of the metal ions (vide Table 8) may be taken as an additional evidence of the above assumption. Another point worth noting is that the affinity of the imidazole groups of transfusion gelatin for copper is less than the values cited for the free imidazole groups (vide Table 13). Such a behaviour is quite understandable in view of the low histidine content of transfusion gelatin[28].

The data on intrinsic affinity constant obtained by means of different techniques have been summarised in the following table

Method	log K	
	Carboxyl groups	imidazole groups
pH metric equilibrium - dialysis	2.10 2.200	3.30 3.40
Polarographic[29]	2.10	-

Zinc and Cadmium-transfusion gelatin systems

The interaction of Zn and Cd with transfusion gelatin is characteristically different in the neutral pH region; although at pH 5.5, both the metal ions show a definite binding with carboxyl groups. In the vicinity of pH 6 and above, zinc appears to bind with the imidazole groups. Rao and Lal have shown in case of bovine serum albumin[30], that copper and zinc combined with the carboxyl groups as well as with the imidazole groups, and cadmium entered into combination only with the free carboxyl groups. The present equilibrium dialysis data exhibit the same behaviour for all the metal ions under discussion. The data listed in table (13) point towards an altogether different behaviour. On the basis of intrinsic affinity, cadmium would be expected to combine with the imidazole groups of transfusion gelatin. With the increase in pH the value of V_M increases (from 8 to 9.7 for a change of pH 5.4 to 7.0 for total metal concentration of 3.9606×10^{-3}M) for zinc transfusion gelatin system, where as for cadmium-transfusion gelatin system V_M increases from 7.6 to 7.9 (for a change of pH 5.4 to 7.0 for total metal concentration 3.368×10^{-3}M). Such data indicate clearly that nearly two zinc ions are bound to the imidazole groups, at a given specific metal concentration, while cadmium shows very little tendency for binding to the imidazole groups. For the time being no plausible explaination can be offered

to such an anamolous behaviour. It may, however, be visualised, that due to numerical prepondrance the available imidazole groups for interaction are in such a low concentration that they can not compete with the large number of freely available carboxyl groups for cadmium binding, as compared to the zinc binding, since the affinity of cadmium for carboxyl groups seems to be greater than that of zinc (Table 13).

The data on intrinsic affinity obtained by two different methods (pH metric and equilibrium dialysis) for the above two systems are summarised in the following table:

Methods	log K(Carboxyl groups)		log K (imidazole groups)	
	Zn	Cd	Zn	Cd
pH-metric	1.87	1.76	2.74	-
Equilibrium dialysis	1.87	1.96	2.91	-

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P A R T II.

Qualitative studies on the interaction of metals with transfusion gelatin and bovine serum albumin by spectrophotometric method.

INTRODUCTION

Job's method[1] of continuous variation has found its most profound manifestation in the spectrophotometric studies of co-ordination compounds. A few references on the feasibility of using conductometry, refractivity[2], viscometry and heat content data for this method are also available, but their scope is limited and in many cases, controversy exists regarding the theoretical propriety of using these properties. Many modification and improvements[3-6] to the method, e.g., Vosburgh and Cooper's method for systems involving more than one complexes[3]; Harvey and Nannings[4] 'Slope Ratio'; and Yoe and Jones[5] 'Molar Ratio' methods point towards its universality in the study of coordination compounds. Inspite of all the merits, the method described above finds limited use in complex systems; like metal-protein complexes, where it is difficult to interpret the spectrophotometric results in terms of stability constant. The difficulty is apparent even in the case of simple molecule, glycyl glycine where disparity exists in the conclusion arrived at, on the basis of different instrumental techniques. Thus for the reactions of copper with glycine and glycyl glycine, Klotz and Co-workers[7] found that the extinction coefficient of the dipeptide was over twice as large as that of the amino acid, while Monk[8] and Li and

Doody[9] on the basis of potentiometric and polarographic investigations showed that copper combined with both the compound to the same extent.

In connection with the use of spectrophotometry in metal-protein system, it will be worthwhile to make a brief mention of the work on the interactions of small molecules with proteins (Warner[10], Plekhan[11], Sone and Co-worker and Klotz). Thus considering the interaction of azodyes with serum albumin, Klotz[13] was able to show that the absorption studies may be successfully employed in calculating the extent of dye-protein interaction with the help of the relationship.[13]

$$\log \frac{Io}{I} = E_p C_F d - E_B C_B d \quad \ldots\ldots\ldots (i)$$

where the subscripts F and B refer to unbound and bound molecule of the dye, E and C refer to molar extinction coefficient and concentration, d the depth of the light path Io and I are the intensities of the light path emerging from pure solvent and solution respectively. The apparent value of E_{app} is given by

$$\log \frac{Io}{I} = E_{app} (C_F - C_B) d \quad \ldots\ldots\ldots (ii)$$

From equation (i) and (ii)

$$x = \frac{E_{app} \times E_B}{E_F - E_B} \quad \ldots\ldots\ldots (iii)$$

where x is the fraction of the dye which is unbound. Hence, to calculate the extent of dye combination, it is necessary

to carryout measurements (i) in absence of protein; (ii) in presence of small quantities of protein (partially unbound) and (iii) in presence of increasing amount of protein (where E_B may be computed).

Equations (i) and (ii) although applicable to protein dye systems have not found general acceptance for metal-protein systems, since complications arise in evaluating separately the extinction coefficient for each type of complex formed in such cases. Nevertheless some workers have employed this technique to elucidate the nature of the metal-protein binding. Klotz and co-workers were able to show the nature of cupric ion binding to several proteins, viz., serum albumin, β Lactoglobulin, γ-globulin lysozyme and casein. Fraenkel - conret[18] and Warner and Weber[10] studied the iron complexes of conalbumin. Mehl and co-worker[19] and Rising and co-workers[20] have studied the cupric ion binding to the proteins in strongly basic medium (under biuret conditions)[21-22] Recently Malik and Salahuddin[23,24,25] carried out systematic studies on more complex system, like metal-gelatin one. Their results, with Cu, Co and Ni (employing transfusion gelatin as a typical well characterised gelatin) gave some valuable informations regarding the mode and extent of combination of the metals to different available sites. It was, therefore, thought worthwhile to extend their studies to some other metal ions also.

Chromium ions are known to play an important role in chrome tanning. Although the Cr(iii) - gelatin system was studied previously by Gustavson[26-27]) and Kuntzels[28-29] Their investigations were limited to the proteins which were not well characterised in terms of hydrogen ion equilibria and molecular weight. Therefore, it was thought necessary to reconsider the problem more critically employing a simpler variety of gelatin, viz., transfusion gelatin. In these studies, besides investigating influence of factors like, concentration of reactants, pH and ionic strength on the extent of binding of Cr(iii) to transfusion gelatin a comparitive study with serum albumin has also been made.

The büiret reaction of copper and nickel with transfusion gelatin has also been studied, and the influence of bivalent metal ions like, Cu, Zn, Cd, Co and Pb on the absorption sprectrum of Ni-transfusion gelatin biuret complex has been investigated.

EXPERIMENTAL

Apparatus

Light absorption measurements were carried out by Backman DU spectrophotometer using tungsten lamp as the light source and corex cell (1 cm. depth). In the latter investigations e.g. in case of biuret reaction, Bausch and Lomb spectromic 20 was used. Backman Model G. pH meter was used for measurement of pH.

Solutions and reagents

Transfusion gelatin (concentration 6%) and crystallised bovine serum albumin (donated by Dr. R.C. Kapoor) were used. Solution of bovine serum albumin was prepared by direct weighing.

Chemically pure (E. Merck) samples of cupric chloride Zinc sulphate, Cadmium sulphate, lead nitrate, cobalt chloride, nickel chloride and chromic chloride (Baker A.R.) were dissolved in triply distilled water (distilled in all glass apparatus) to get their respective solutions. Metal content of the stock solutions were determined as described earlier by complexometric titration[30], and for the determination of chromium content, the spectrophotometric method recommended by Green and Aug.[31] was adopted. A.R. potassium

hydroxide and potassium chloride were used to prepare their solutions. These solutions were used to maintain the constant pH and ionic strength respectively. Solutions of EDTA (E. Merck), E. Black T. and Murexide were prepared as recommended by Schwarzenbach and Irving.[30] Walpole acetate buffers[32] were prepared from 0.2 M solutions of acetic acid and sodium acetate and their pH values were checked.

Nickel-transfusion gelatin biuret complex was prepared by mixing equal volumes of nickel chloride (dilute solution) and transfusion gelatin. The pH of the mixture was adjusted to 12.3 and heated slightly (35 to 40°C) for about half an hour and than the resulting solution (orange coloured) was filtered off, the nickel hydroxide precipitate was washed repeatedly, with warm dil. alkali (pH 12.0) until the washing were free from protein. The complex was stored in pyrex glass bottles and nickel content was determined.

Procedure

(i) Chromic chloride (concentration given in tables) and transfusion gelatin (1.8%) were mixed in a number of pyrex boiling tubes. Their pH values were adjusted to 3.7, 4.4, 4.8, 5.2, 5.57 and 5.8 by the addition of buffers. The ionic strength was maintained at 0.4 by adding requisite amount of potassium chloride. Another set in which the pH values were adjusted by the addition of dilute potassium

hydroxide instead of the buffers was also analysed spectrophotometrically.

(ii) At a fixed pH (5.57) and ionic strength (0.4) two sets of mixtures were prepared. In one the chromium concentration was kept fixed and the concentration of the protein was varied while in the other there was fixed amount of protein and the concentration of the metal was changed.

(iii) Mixtures were analysed spectrophotometrically at three different ionic strength viz, 0.5, 0.4 and 0.2 taking varying concentration of metal ion with a fixed amount of protein (1.8%),

B. Mixtures of chromic chloride and bovine serum albuman were prepared under strictly identical conditions and analysed spectrophotometrically.

C. (i) Cupric Chloride (1.0×10^{-3}M) and transfusion gelatin (0.6%) were mixed in a number of pyrex test tubes. Their pH values were adjusted to 5.4, 6.8, 8.9, 10.5, 11.4, 11.8, 12.0, 12.2 and 12.4 by the addition of dilute potassium hydroxide solution. The ionic strength was maintained at 0.15 by adding requisite amount of potassium chloride, and total volume was made upto 10 m.l.

(ii) At a fixed pH (12.0) and ionic strength (0.15) two sets of mixtures were prepared. In one the cupric chloride concentration was varied and protein concentration was kept fixed while in the other there was fixed amount of metal

ion and the concentration of protein was changed.

(iii) At fixed pH (12.0), metal (1.0×10^{-3} M) and protein (0.6%) were mixed in different pyrex test tubes, and varying amounts of potassium chloride were added as to maintain the different ionic strength viz., 0.075, 0.15, 0.225, 0.3, 0.45 and 0.6.

D. Mixtures containing nickel chloride and bransfusion gelatin were prepared at different pH values (for the fixed ionic strength, metal and protein concentration), at varying concentration of metal ions (for the fixed ionic strength pH and protein concentration), at varying concentration of protein (for the fixed pH, ionic strength and metal concentration), and at varying ionic strength (for the fixed pH, metal and protein concentration).

E. 2 m.l. of Ni-transfusion gelatin biuret complex was mixed with varying concentrations of copper, zinc, cadmium, lead and cobalt in different pyrex test tubes. The pH of all the mixtures were adjusted to 12.0 and ionic strength at 0.15 by the addition of requisite amount of potassium chloride and total volume was made upto 10 m.l. The optical density of all the mixtures were measured along with the blank solution in which only 2 m.l. of Ni-transfusion gelatin biuret complex was diluted to 10 m.l. adjusting the pH at 12.0 and ionic strength at 0.15, thus the observations were made on the effect of copper, zinc, cadmium, lead and cobalt (at valuing concentration) on the absorption spectrum of Ni-transfusion gelatin biuret complex.

T A B L E 1.

Concentration of Chromic Chloride = 1×10^{-2} M

Concentration of transfusion gelatin = 1.8 %

Total volume 10 m.l. ionic strength = 0.4

Wave Length (mu)	O.D. at pHs (Acetate buffers)		
	pH 3.7	pH 4.4	pH 4.8
375	0.275	0.280	0.300
400	0.285	0.287	0.310
410	0.295	0.300	0.320
420	0.290	0.292	0.315
430	0.267	0.270	0.296
450	0.194	0.196	0.224
475	0.112	0.115	0.133
500	0.083	0.086	0.102
525	0.120	0.125	0.143
550	0.190	0.200	0.223
560	0.215	0.228	0.252
570	0.234	0.247	0.268
580	0.242	0.254	0.277
590	0.238	0.252	0.274
600	0.228	0.240	0.260
625	0.175	0.182	0.197
650	0.113	0.115	0.124

(Fig. 1)

T A B L E 2.

Concentration of Chromic Chloride = 1×10^{-2} M

Concentration of transfusion gelatin = 1.8 %

Total volume 10 m.l. ionic strength = 0.4

Wave Length (mu)	O.D. at pHs (Acetate buffer)		
	pH 5.2	pH 5.57	pH 5.8
375	0.285	0.287	0.295
400	0.308	0.307	0.313
410	0.322	0.320	0.326
420	0.316	0.320	0.325
430	0.298	0.302	0.306
450	0.226	0.230	0.235
475	0.135	0.135	0.136
500	0.104	0.096	0.102
525	0.145	0.144	0.145
550	0.230	0.234	0.239
560	0.260	0.265	0.269
570	0.280	0.290	0.295
580	0.290	0.300	0.304
590	0.286	0.294	0.298
600	0.268	0.280	0.284
625	0.204	0.208	0.215
650	0.130	0.134	0.135

(Fig. 2.)

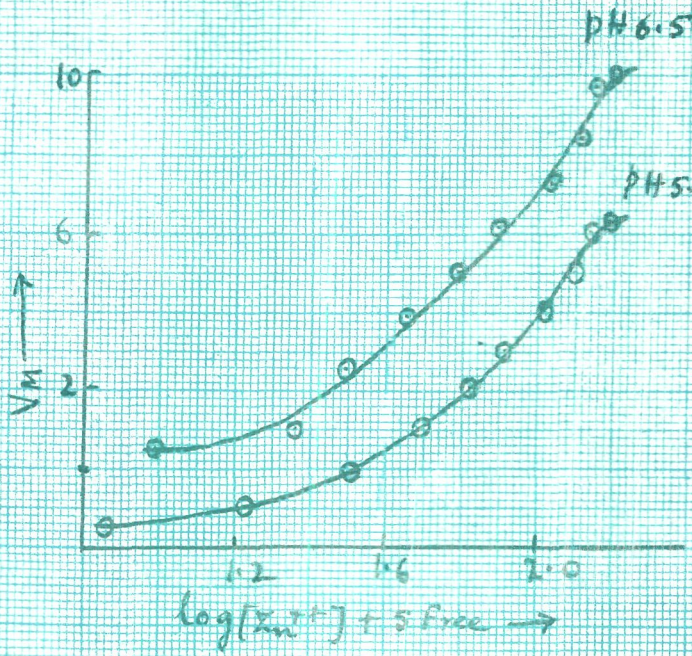


FIG-2 Binding of Zn^{++} by transfusion gelatin

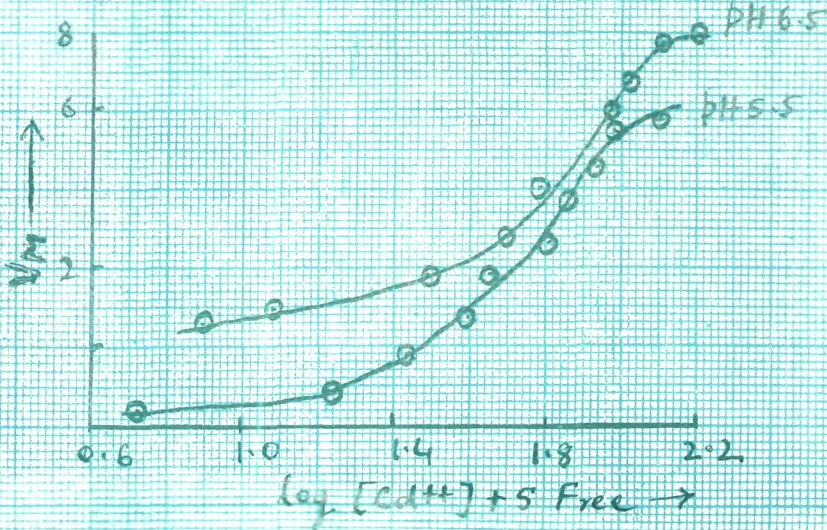


FIG-3 Binding of Cd^{++} by transfusion gelatin.

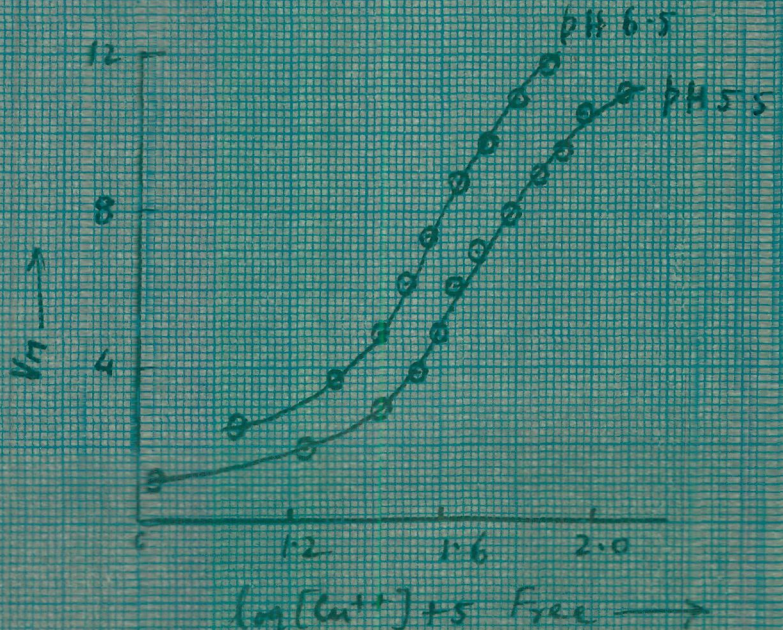


Fig 1 Binding of Cu^{++} by Transforum gelatin

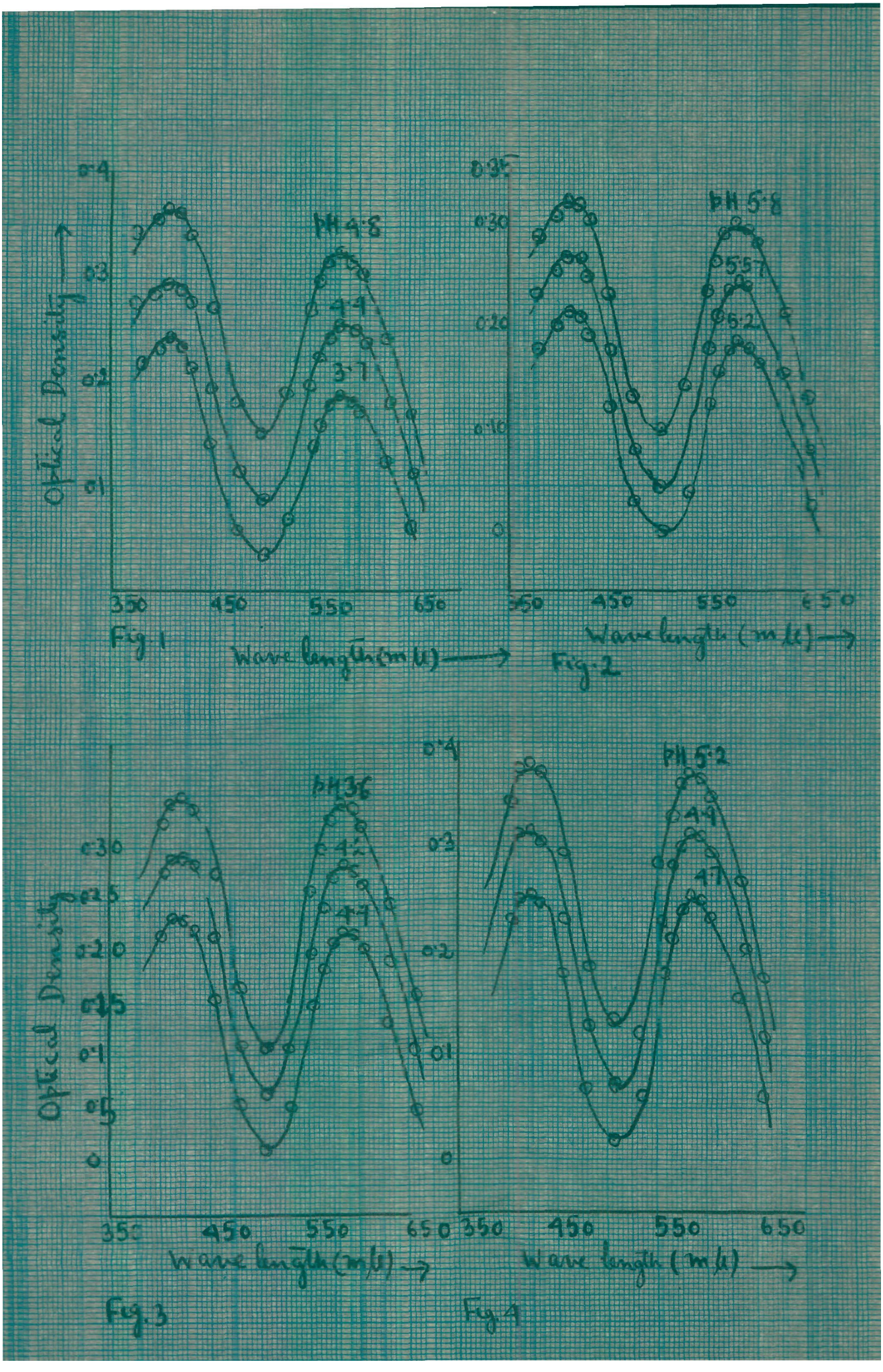


Fig. 1

Fig. 2

Fig. 3

Fig. 4

TABLE 3.

Concentration of Chromic Chloride = 1×10^{-2} M

Concentration of transfusion gelatin = 1.8 %

Total volume 10 m.l. ionic strength = 0.4

Wave Length (mu)	O.D. at pHs (KOH)		
	pH 3.6	pH 4.2	pH 4.4
375			
400	0.317	0.328	0.329
410	0.338	0.346	0.347
420	0.334	0.346	0.350
430	0.325	0.335	0.340
450	0.256	0.266	0.275
475	0.155	0.163	0.166
500	0.110	0.113	0.115
525	0.152	0.158	0.158
550	0.250	0.261	0.262
560	0.286	0.297	0.300
570	0.312	0.324	0.329
580	0.325	0.335	0.343
590	0.322	0.332	0.340
600	0.305	0.316	0.324
625	0.234	0.243	0.247
660	0.148	0.159	0.155

(Fig. 3.)

TABLE 4.

Concentration of Chromic Chloride = 1×10^{-2} M

Concentration of transfusion gelatin = 1.8 %

Total volume 10 m.l. ionic strength = 0.40

Wave Langth (mu)	O.D. at pHs (KOH)		
	pH 4.7	pH 4.9	pH 5.2
400	0.332	0.350	0.354
410	0.354	0.363	0.376
420	0.356	0.367	0.380
430	0.345	0.354	0.366
450	0.275	0.284	0.294
475	0.170	0.175	0.181
500	0.117	0.120	0.128
525	0.164	0.168	0.176
550	0.276	0.282	0.285
560	0.315	0.334	0.330
570	0.340	0.350	0.365
580	0.354	0.365	0.372
590	0.350	0.362	0.365
600	0.335	0.342	0.345
625	0.250	0.252	0.260
650	0.160	0.165	0.170

(Fig. 4.)

TABLE 5.

Concentration of Chromic Chloride = 1×10^{-2} M

pH (Acetate buffer) = 5.57

Total volume 20 m.l. ionic strength = 0.4

Wave Length (mu)	O.D.		
	0 % protein	0.6 % protein	0.9 % protein
375	0.150	0.170	0.190
400	0.158	0.175	0.195
410	0.170	0.200	0.212
420	0.167	0.192	0.214
430	0.154	0.177	0.203
450	0.102	0.126	0.148
475	0.048	0.067	0.085
500	0.046	0.052	0.063
525	0.068	0.074	0.085
550	0.112	0.122	0.135
560	0.130	0.139	0.154
570	0.143	0.152	0.172
580	0.148	0.162	0.177
590	0.152	0.162	0.177
600	0.145	0.153	0.172
625	0.116	0.124	0.137
650	0.075	0.081	0.092

(Fig. 5.)

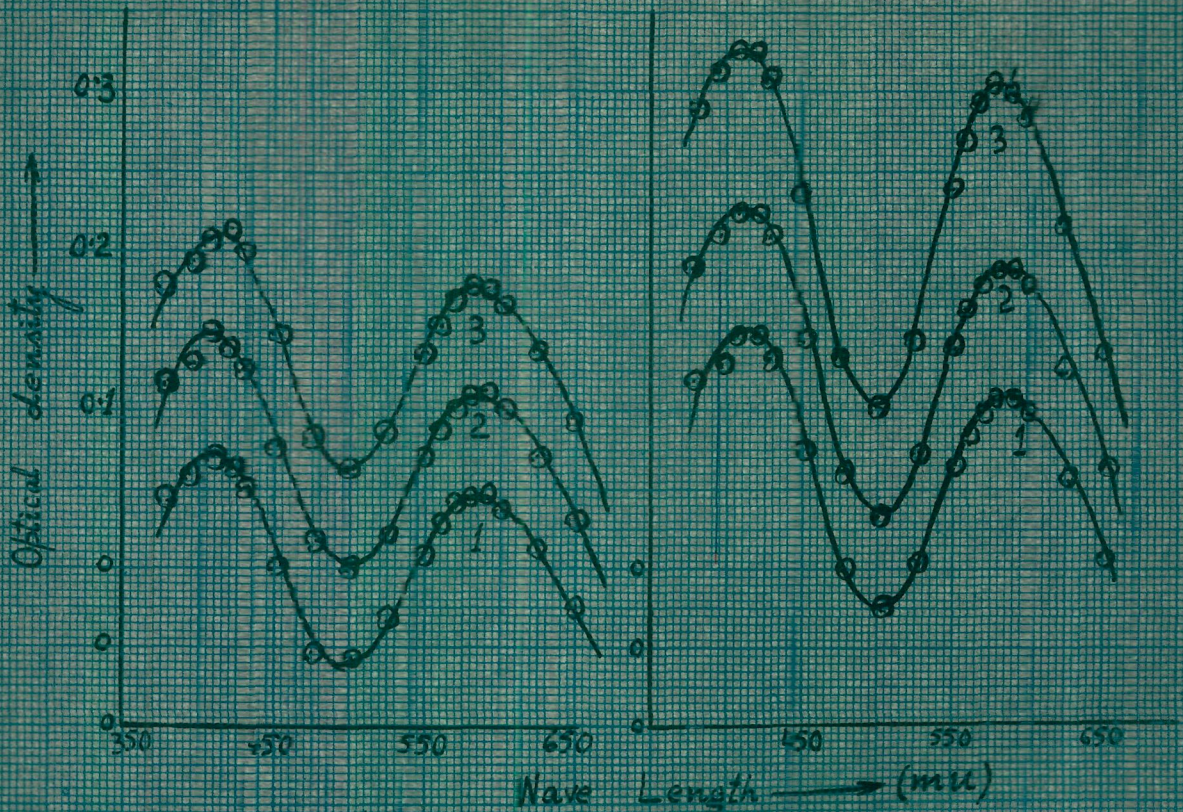


Fig 5, (1) → 2% (2) → 0·6% and (3) → 0·9% protein

Fig 6, (1) → 1·2% (2) → 1·5% and (3) → 1·8% protein

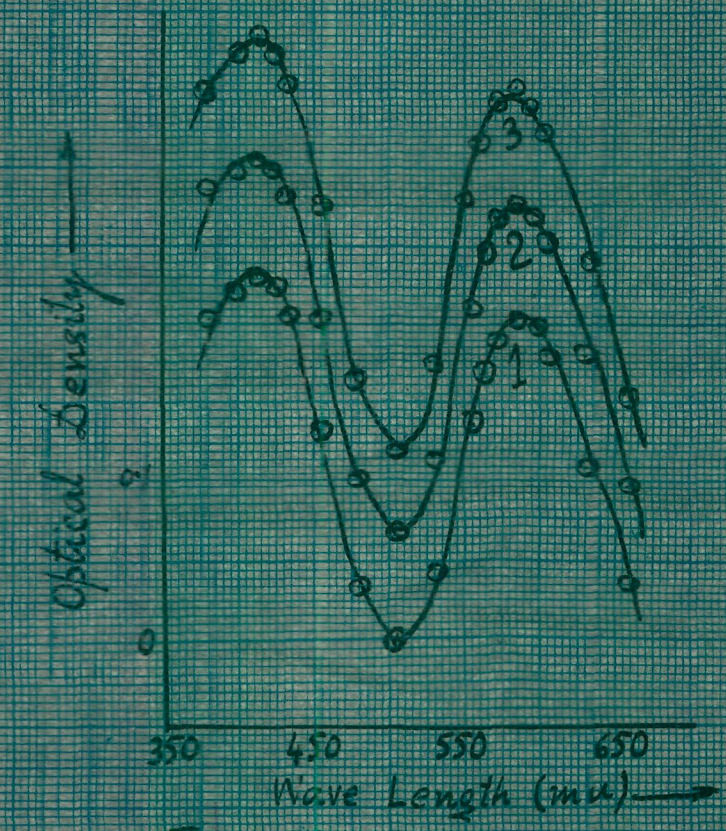


Fig 7
(1) 2·1% (2) 3·5% and (3) 4% protein

TABLE 6.

Concentration of Chromic Chloride = 1×10^{-2} M

pH (Acetate buffer) = 5.57

Total volume 20 m.l. ionic strength = 0.40

Wave Length (mu)	O.D.		
	1.2 % protein	1.5 % protein	1.8 % protein
375	0.220	0.248	0.295
400	0.228	0.258	0.313
410	0.245	0.274	0.326
420	0.246	0.275	0.325
430	0.232	0.257	0.306
450	0.173	0.193	0.235
475	0.098	0.112	0.136
500	0.074	0.083	0.102
525	0.104	0.118	0.145
550	0.164	0.188	0.239
560	0.184	0.214	0.269
570	0.198	0.232	0.295
580	0.208	0.240	0.304
590	0.208	0.240	0.298
600	0.200	0.227	0.284
625	0.157	0.177	0.215
650	0.102	0.113	0.135

(Fig. 6.)

TABLE 7.

Concentration of Chromic Chloride = 1×10^{-2} M

pH (Acetate buffer) = 5.57

Total volume 20 m.l. ionic strength = 0.4

Wave Length (mu)	O.D.		
	2.1 % Protein	3.5 % protein	4.0 % protein
375	0.311	0.340	0.365
400	0.322	0.348	0.372
410	0.332	0.354	0.385
420	0.328	0.352	0.376
430	0.308	0.374	0.357
450	0.232	0.255	0.275
475	0.135	0.153	0.165
500	0.100	0.115	0.122
525	0.147	0.165	0.175
550	0.238	0.264	0.280
560	0.272	0.298	0.315
570	0.291	0.322	0.345
580	0.308	0.330	0.352
590	0.298	0.322	0.345
600	0.280	0.305	0.325
625	0.212	0.232	0.242
650	0.135	0.145	0.152

(Fig. 7.)

TABLE 8.

Concentration of transfusion gelatin = 1.5 %

pH (Acetate buffer) = 5.57

Total volume 10 m.l. ionic strength = 0.4

Wave Length (mu)	O.D.			
	$0.25 \times 10^{-2} M Cr^{+3}$	$0.25 \times 10^{-2} M Cr^{+3}$ + Protein	$0.5 \times 10^{-2} M Cr^{+3}$	$0.5 \times 10^{-2} M Cr^{+3}$ + Protein
375	0.040	0.148	0.070	0.180
400	0.048	0.154	0.078	0.184
410	0.054	0.158	0.090	0.186
420	0.052	0.162	0.089	0.190
430	0.046	0.130	0.083	0.162
450	0.032	0.100	0.054	0.133
475	0.012	0.058	0.027	0.091
500	0.004	0.032	0.022	0.062
525	0.010	0.050	0.035	0.087
550	0.018	0.092	0.057	0.135
560	0.025	0.105	0.066	0.148
570	0.032	0.116	0.073	0.159
580	0.035	0.122	0.076	0.165
590	0.038	0.118	0.078	0.162
600	0.034	0.106	0.074	0.150
625	0.012	0.092	0.062	0.116
650	0	0.050	0.042	0.072

(Fig. 8.)

Curves 1, 3 for 0·25 and 0·5 x 10^{-3} M Cr^{3+}
2, 4, " " " " " " " " + protein

1, 3 for 0·75 and 1·5 x 10^{-3} M Cr^{3+}
2, 4, " " " " " " " " + protein

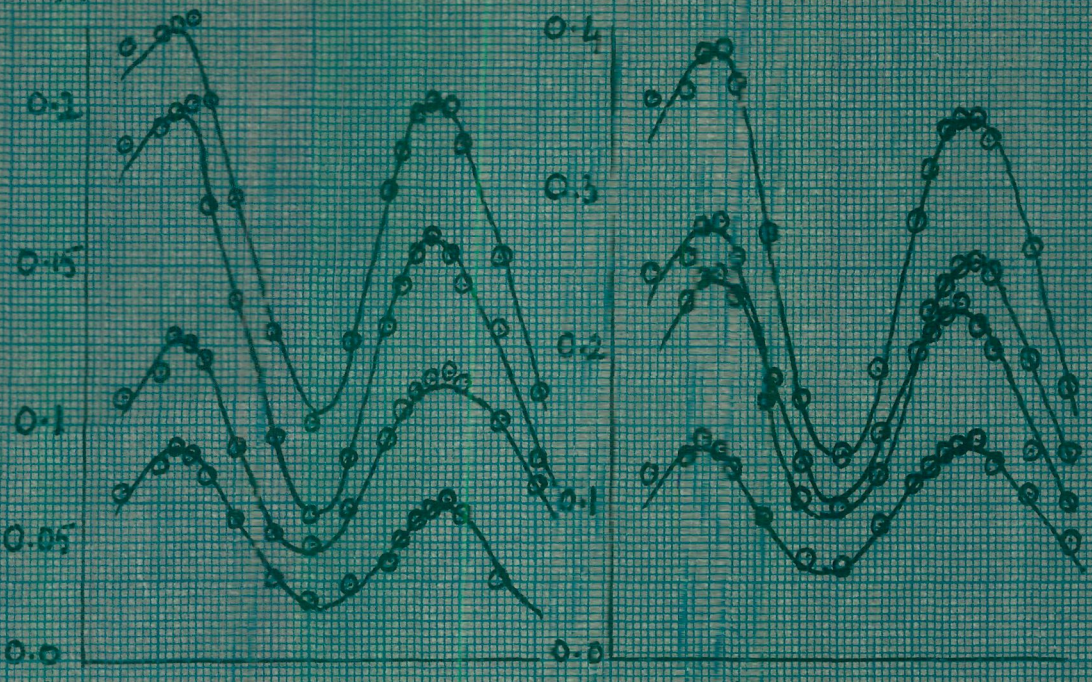


FIG-8. Wave length (mμ) FIG-9. Wave length (mμ)

1 and 3 for 2 and 3 x 10^{-2} M Cr^{3+}
2 " 4 " " " " " " " + protein

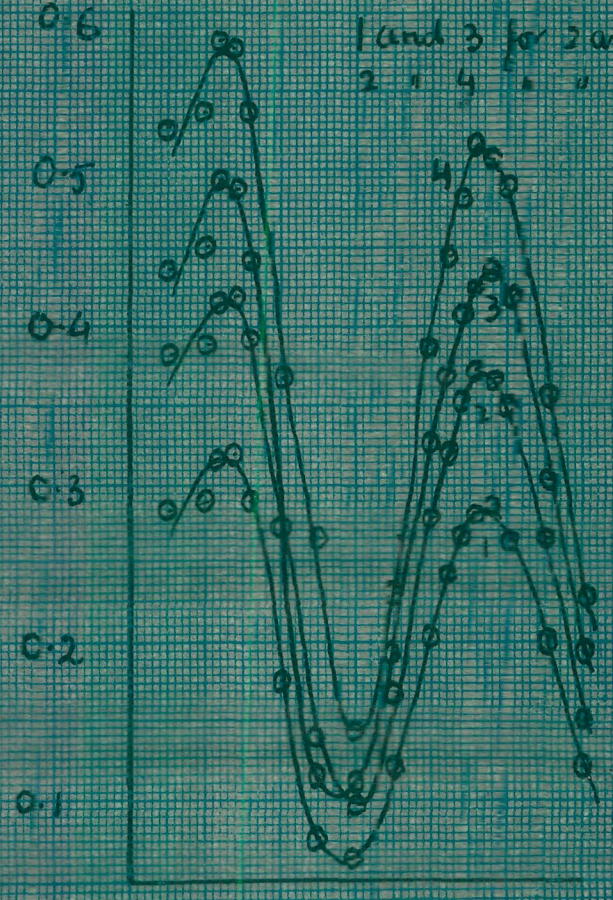


FIG-10 Wave length (mμ)

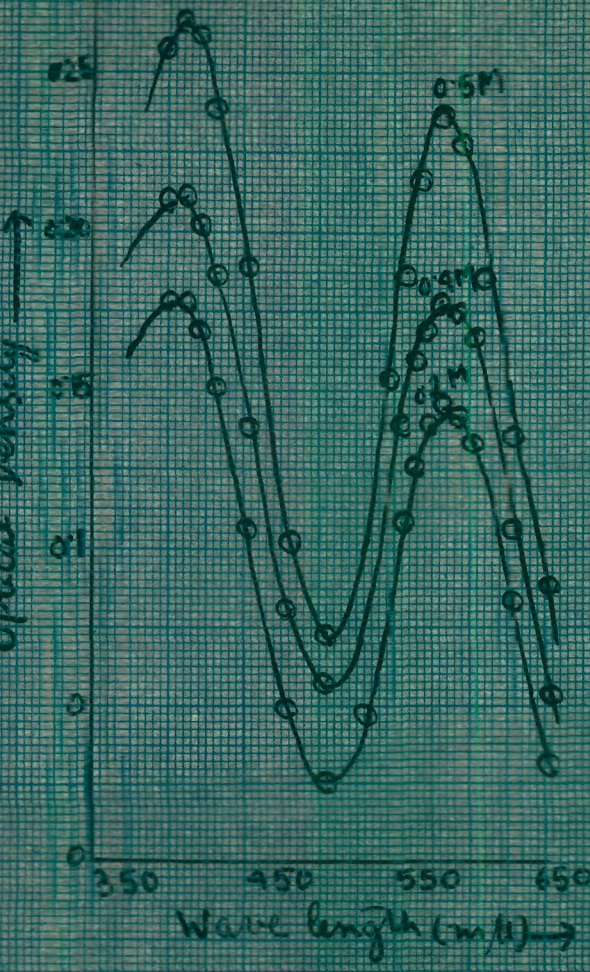


Fig. 11

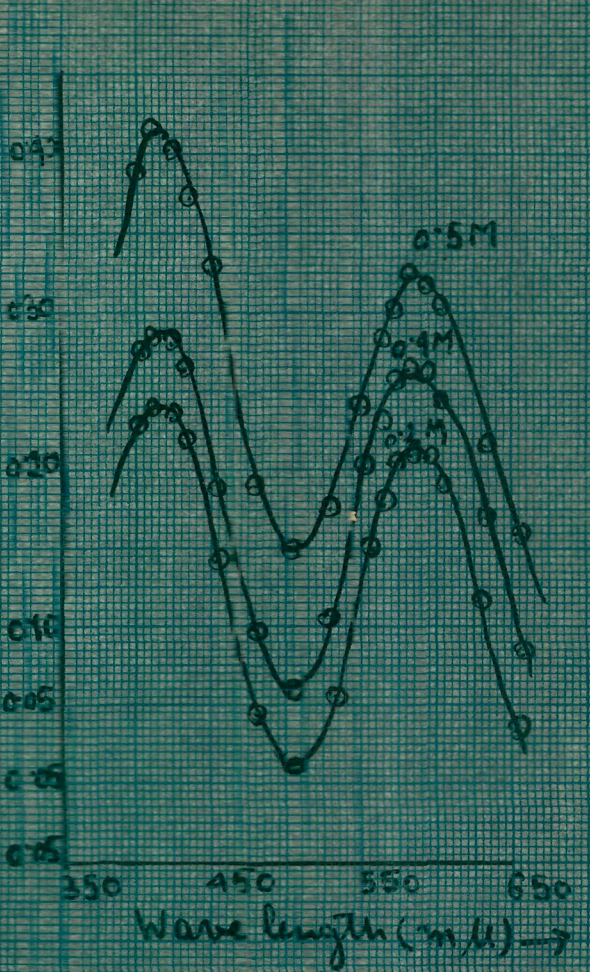


Fig. 12

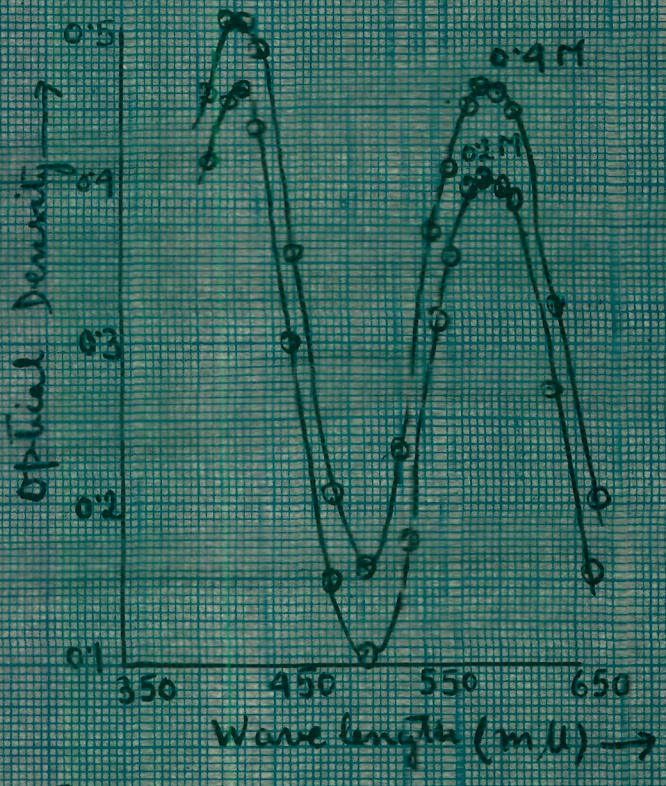


Fig. 13

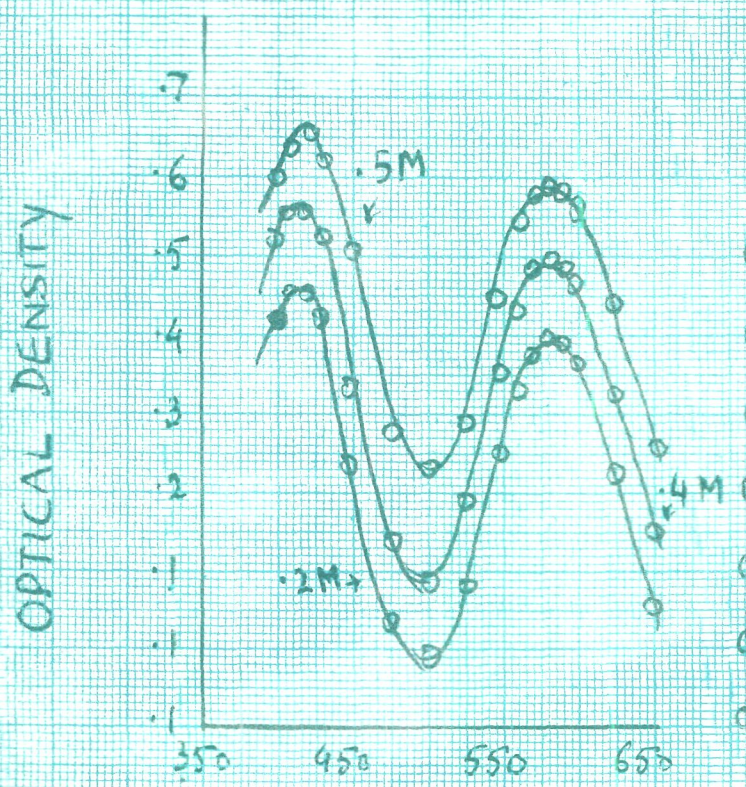


FIG-14 Wave length (mμ)

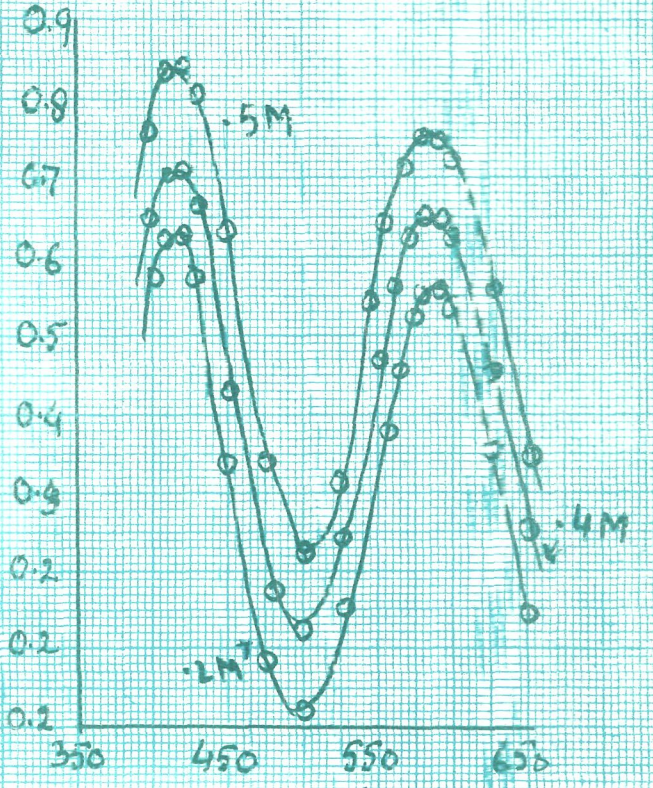


FIG-15 Wave length (mμ)

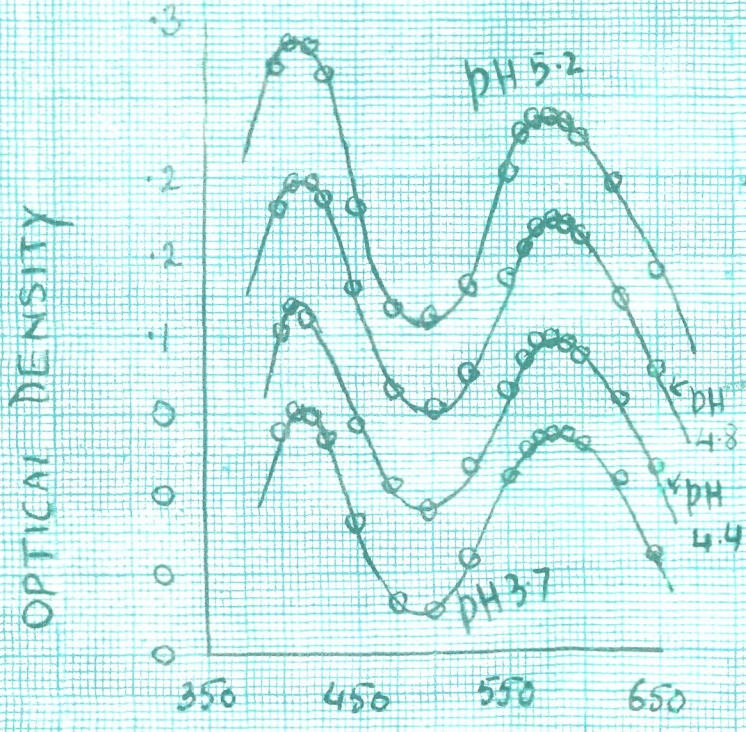


FIG-16 Wave length (mμ)

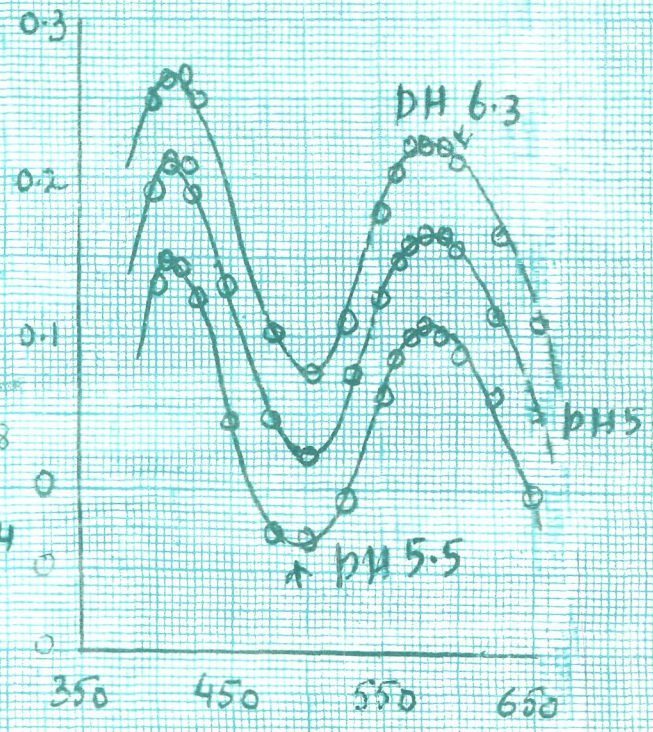


FIG-17 Wave length (mμ)

TABLE 9.

Concentration of transfusion gelatin = 1.5 %

pH (Acetate buffer) = 5.57

Total volume 10 m.l. ionic strength = 0.4

Wave Length (mu)	O.D.			
	$0.75 \times 10^{-2} MCr^{+3}$	$0.75 \times 10^{-2} MCr^{+3}$ + Protein	$1.5 \times 10^{-2} MCr^{+3}$	$1.5 \times 10^{-2} MCr^{+3}$ + Protein
375	0.092	0.118	0.220	0.328
400	0.100	0.202	0.228	0.334
410	0.112	0.218	0.250	0.360
420	0.108	0.220	0.250	0.360
430	0.098	0.204	0.227	0.338
450	0.063	0.140	0.146	0.245
475	0.036	0.098	0.076	0.140
500	0.034	0.072	0.070	0.106
525	0.058	0.116	0.093	0.158
550	0.086	0.168	0.168	0.250
560	0.098	0.182	0.192	0.284
570	0.106	0.194	0.211	0.310
580	0.110	0.201	0.224	0.316
590	0.116	0.119	0.226	0.316
600	0.090	0.106	0.220	0.304
625	0.078	0.086	0.164	0.233
650	0.050	0.072	0.104	0.048

(Fig. 9.)

TABLE 10.

Concentration of transfusion gelatin = 1.5 %

pH (Acetate buffer) = 5.57

Total volume 10 m.l. ionic strength = 0.4

Wave Length	O.D.			
	2×10^{-2} MCr^{+3}	2×10^{-2} MCr^{+3} + Protein	3×10^{-2} MCr^{+3}	3×10^{-2} MCr^{+3} + Protein
375	0.310	0.405	0.465	0.550
400	0.315	0.410	0.472	0.560
410	0.345	0.443	0.518	0.608
420	0.345	0.443	0.512	0.602
430	0.313	0.415	0.462	0.560
450	0.200	0.295	0.295	0.390
475	0.100	0.164	0.144	0.208
500	0.088	0.127	0.132	0.167
525	0.144	0.192	0.214	0.255
550	0.227	0.304	0.344	0.412
560	0.265	0.345	0.390	0.468
570	0.290	0.376	0.432	0.503
580	0.305	0.397	0.452	0.542
590	0.310	0.386	0.460	0.530
600	0.286	0.375	0.440	0.512
625	0.225	0.290	0.336	0.388
650	0.145	0.176	0.220	0.250

(Fig. 10.)

TABLE 11.

Concentration of Chromic Chloride = 0.25×10^{-2}M

Concentration of transfusion gelatin = 1.8 %

Total volume 10 m.l. Unbuffered system

Wave length (mu)	O.D. at ionic strength		
	0.2	0.4	0.5
400	0.233	0.238	0.260
410	0.232	0.238	0.270
420	0.223	0.230	0.265
430	0.206	0.214	0.242
450	0.158	0.166	0.190
475	0.102	0.108	0.090
500	0.076	0.083	0.072
525	0.100	0.106	0.098
550	0.162	0.166	0.155
560	0.178	0.186	0.188
570	0.193	0.197	0.198
580	0.198	0.205	0.240
590	0.196	0.202	0.228
600	0.185	0.192	0.185
625	0.137	0.134	0.135
650	0.083	0.080	0.090
675	0.048	0.045	0.050

(Fig. 11.)

TABLE 12.

Concentration of Chromic Chloride = 1.0×10^{-2} M

Concentration of transfusion gelatin = 1.8 %

Total volume 10 m.l. Unbuffered system

Wave Length (mu)	O.D. at ionic strength		
	0.2	0.4	0.5
400	0.332	0.328	0.390
410	0.342	0.338	0.422
420	0.340	0.336	0.405
430	0.320	0.316	0.370
450	0.245	0.240	0.330
475	0.146	0.144	0.195
500	0.112	0.110	0.180
525	0.156	0.155	0.150
550	0.254	0.253	0.240
560	0.284	0.284	0.288
570	0.310	0.312	0.300
580	0.315	0.316	0.325
590	0.312	0.314	0.322
600	0.293	0.295	0.305
625	0.218	0.220	0.215
650	0.134	0.135	0.160
675	0.078	0.078	0.098

(Fig. 12.)

TABLE 13.

Concentration of Chromic Chloride = 2.5×10^{-2} M

Concentration of transfusion gelatin = 1.8 %

Total volume 10 m.l. Unbuffered system

Wave Length (mu)	O.D. at ionic strength	
	0.2	0.4
400	0.466	0.460
410	0.518	0.512
420	0.515	0.510
430	0.490	0.492
450	0.354	0.354
475	0.204	0.205
500	0.155	0.158
525	0.228	0.232
550	0.368	0.373
560	0.407	0.412
570	0.450	0.456
580	0.457	0.465
590	0.452	0.462
600	0.447	0.450
625	0.326	0.329
650	0.208	0.206
675	0.120	0.117

(Fig. 13.)

TABLE 14.

Concentration of Chromic Chloride = 3.0×10^{-2} M

Concentration of transfusion gelatin = 1.8 %

Total volume 10 m.l. Unbuffered system

Wave Length (mu)	O.D. at ionic strength		
	0.2	0.4	0.5
400	0.630	0.630	0.610
410	0.673	0.673	0.650
420	0.672	0.672	0.675
430	0.630	0.630	0.640
450	0.448	0.448	0.520
475	0.248	0.250	0.290
500	0.195	0.198	0.245
525	0.294	0.298	0.295
550	0.462	0.464	0.460
560	0.540	0.543	0.550
570	0.593	0.596	0.590
580	0.615	0.609	0.603
590	0.608	0.600	0.596
600	0.580	0.588	0.590
625	0.438	0.435	0.450
650	0.270	0.268	0.270
675	0.152	0.148	0.150

(Fig. 14.)

T A B L E 15.

Concentration of Chromic Chloride = 4.0×10^{-2}M

Concentration of transfusion gelatin = 1.8 %

Total volume 10 m.l. Unbuffered system

Wave Length (mu)	O.D. at ionic strength		
	0.2	0.4	0.5
400	0.780	0.760	0.775
410	0.842	0.822	0.850
420	0.842	0.825	0.850
430	0.778	0.770	0.820
450	0.547	0.540	0.640
475	0.290	0.286	0.350
500	0.230	0.225	0.232
525	0.356	0.350	0.320
550	0.586	0.578	0.550
560	0.672	0.668	0.645
570	0.742	0.735	0.720
580	0.780	0.768	0.760
590	0.778	0.760	0.756
600	0.748	0.740	0.732
625	0.570	0.562	0.561
650	0.352	0.360	0.362
675	0.206	0.295	0.290

(Fig. 15.)

TABLE 16.

Concentration of Chromic Chloride = 1×10^{-2}M

Concentration of bovine serum albumin = 1.0 %

Total volume 10 m.l. ionic strength = 0.15

Wave Length	O.D. at pHs (Acetate buffer)			
	pH 3.7	pH 4.4	pH 4.8	pH 5.2
400	0.148	0.163	0.192	0.228
410	0.164	0.178	0.210	0.248
420	0.160	0.171	0.208	0.246
430	0.144	0.156	0.200	0.224
450	0.090	0.104	0.140	0.142
475	0.040	0.064	0.074	0.074
500	0.036	0.044	0.063	0.068
525	0.068	0.076	0.085	0.091
550	0.118	0.126	0.145	0.164
560	0.137	0.145	0.164	0.188
570	0.148	0.156	0.180	0.195
580	0.152	0.158	0.183	0.198
590	0.150	0.154	0.180	0.192
600	0.148	0.150	0.178	0.186
625	0.116	0.120	0.134	0.162
650	0.072	0.078	0.090	0.100

(Fig. 16.)

TABLE 17.

Concentration of Chromic Chloride = 1.0×10^{-2} M

Concentration of bovine serum albomin = 1.0 %

Total volume 10 m.l. ionic strength = 0.15

Wave Length (mu)	O.D. at pHs (Acetate buffer)		
	pH 5.5	pH 5.9	pH 6.3
400	0.240	0.252	0.258
410	0.262	0.273	0.274
420	0.258	0.270	0.275
430	0.236	0.251	0.256
450	0.154	0.188	0.192
475	0.082	0.108	0.110
500	0.078	0.080	0.083
525	0.101	0.133	0.116
550	0.170	0.182	0.185
560	0.195	0.208	0.210
570	0.206	0.218	0.228
580	0.216	0.226	0.230
590	0.210	0.222	0.226
600	0.198	0.219	0.220
625	0.170	0.170	0.172
650	0.104	0.110	0.110

(Fig. 17.)

TABLE 18.

Concentration of Chromic Chloride = 1.0×10^{-2} M

Concentration of bovine serum albumin = 1.0 %

Total volume 10 m.l. ionic strength = 0.15

Wave Length	O.D. at pHs (KOH)		
	pH 3.6	pH 4.1	pH 4.4
400	0.265	0.262	0.267
410	0.274	0.280	0.285
420	0.271	0.270	0.278
430	0.246	0.248	0.252
450	0.175	0.174	0.178
475	0.092	0.095	0.100
500	0.062	0.066	0.064
525	0.102	0.106	0.108
550	0.171	0.180	0.185
560	0.205	0.207	0.212
570	0.214	0.220	0.228
580	0.220	0.225	0.235
590	0.218	0.220	0.230
600	0.206	0.210	0.218
625	0.155	0.160	0.162
650	0.093	0.090	0.098

(Fig. 18.)

TABLE 19.

Concentration of Chromic Chloride = 1.0×10^{-2} M

Concentration of bovine serum albumin = 1.0 %

Total volume 10 m.l. ionic strength = 0.15

Wave Length (mu)	O.D. at pHs (KOH)		
	pH 4.65	pH 4.8	pH 5.15
400	0.286	0.290	0.308
410	0.298	0.300	0.316
420	0.290	0.195	0.310
430	0.269	0.276	0.292
450	0.198	0.204	0.220
475	0.110	0.113	0.130
500	0.080	0.082	0.100
525	0.121	0.123	0.138
550	0.195	0.204	0.219
560	0.224	0.231	0.248
570	0.240	0.246	0.265
580	0.248	0.259	0.270
590	0.242	0.253	0.264
600	0.230	0.240	0.255
625	0.170	0.176	0.193
650	0.100	0.104	0.120

(Fig. 19)

T A B L E 20.

Concentration of Chromic Chloride = 1.0×10^{-2} M

Total volume 10 m.l. ionic strength = 0.15

pH = 5.5

Wave Length (mu)	O.D. at protein concentration		
	0	0.25 %	0.5 %
400	0.158	0.166	0.182
410	0.170	0.178	0.202
420	0.167	0.176	0.198
430	0.154	0.164	0.188
450	0.102	0.112	0.132
475	0.048	0.054	0.068
500	0.046	0.052	0.060
525	0.068	0.074	0.082
550	0.112	0.120	0.136
560	0.130	0.137	0.160
570	0.143	0.151	0.167
580	0.148	0.156	0.174
590	0.152	0.154	0.170
600	0.145	0.150	0.162
625	0.116	0.122	0.140
650	0.075	0.080	0.090

(Fig. 20.)

TABLE 21.

Concentration of Chromic Chloride = 1.0×10^{-2}M

Total volume 10 m.l. ionic strength = 0.15

pH 5.5

Wave Length (mu)	O.D. at protein concentration		
	0.75 %	1.25 %	1.5 %
400	0.200	0.224	0.238
410	0.228	0.250	0.270
420	0.224	0.246	0.265
430	0.202	0.230	0.248
450	0.140	0.152	0.170
475	0.072	0.080	0.098
500	0.067	0.073	0.090
525	0.090	0.096	0.112
550	0.151	0.171	0.192
560	0.176	0.206	0.216
570	0.186	0.218	0.226
580	0.198	0.226	0.232
590	0.193	0.220	0.247
600	0.184	0.210	0.236
625	0.160	0.182	0.202
650	0.100	0.120	0.150

(Fig. 21.)

TABLE 22.

Concentration of bovine serum albumin = 1.0 %

Total volume 10 m.l. ionic strength = 0.15

pH 5.5

Wave Length (mu)	O.D. at metal concentration ($x10^{-2}$M)			
	0.5	0.5 + Protein	1.5	1.5 + Protein
400	0.074	0.192	0.226	0.268
410	0.091	0.214	0.250	0.304
420	0.086	0.210	0.248	0.300
430	0.082	0.195	0.226	0.280
450	0.053	0.118	0.145	0.202
475	0.026	0.050	0.079	0.136
500	0.020	0.040	0.072	0.130
525	0.034	0.062	0.094	0.154
550	0.053	0.100	0.169	0.223
560	0.066	0.124	0.192	0.258
570	0.072	0.130	0.210	0.266
580	0.076	0.145	0.224	0.272
590	0.079	0.138	0.228	0.264
600	0.073	0.126	0.220	0.260
625	0.060	0.098	0.162	0.132
650	0.040	0.050	0.104	0.066

(Fig. 22.)

TABLE 23.

Concentration of bovine serum albumin = 1.0 %

Total volume 10 m.l. ionic strength = 0.15

pH 5.5

Wave Length (mu)	O.D. at metal concentration ($x10^{-2}$M)			
	2.0	2.0 + Protein	3.0	3.0 + Protein
400	0.316	0.326	0.470	0.481
410	0.346	0.358	0.516	0.490
420	0.345	0.354	0.510	0.485
430	0.310	0.335	0.463	0.466
450	0.200	0.270	0.296	0.402
475	0.098	0.210	0.143	0.348
500	0.086	0.201	0.133	0.338
525	0.145	0.222	0.212	0.360
550	0.226	0.292	0.342	0.438
560	0.266	0.323	0.389	0.470
570	0.290	0.330	0.432	0.477
580	0.305	0.336	0.452	0.482
590	0.310	0.328	0.461	0.475
600	0.285	0.322	0.440	0.468
625	0.224	0.258	0.332	0.345
650	0.143	0.192	0.218	0.250

(Fig. 23.)

TABLE 24.

Concentration of Chromic Chloride = 1.0×10^{-2} M

Concentration of bovine serum albumin = 1.0 %

Total volume 10 m.l. pH 5.5

Wave Length (mu)	O.D. at ionic strength		
	0.2	0.4	0.5
400	0.240	0.250	0.252
410	0.263	0.272	0.274
420	0.260	0.270	0.271
430	0.238	0.248	0.250
450	0.154	0.187	0.188
475	0.083	0.106	0.109
500	0.078	0.081	0.080
525	0.102	0.130	0.134
550	0.171	0.180	0.184
560	0.196	0.209	0.210
570	0.208	0.219	0.220
580	0.220	0.226	0.228
590	0.212	0.224	0.220
600	0.200	0.218	0.219
625	0.170	0.169	0.172
650	0.105	0.108	0.110

(Fig. 24.)

TA B L E 25.

Concentration of Cupric Chloride = 1.0×10^{-3}M

Concentration of transfusion gelatin = 0.6 %

Total volume 10 m.l. ionic strength = 0.15

Wave Length (mu)	O.D. at pHs (KOH)				
	5.4	6.8	8.9	10.5	11.4
400	0.070	0.070	0.070	0.100	0.090
450	0.030	0.030	0.050	0.090	0.088
500	0.020	0.028	0.080	0.140	0.140
525	0.010	0.035	0.100	0.150	0.160
550	0.020	0.060	0.120	0.165	0.170
565	0.020	0.065	0.125	0.162	0.150
575	0.020	0.070	0.110	0.130	0.140
600	0.030	0.080	0.108	0.120	0.115
625	0.040	0.095	0.070	0.080	0.080
650	0.050	0.075	0.068	0.040	0.030

Wave Length (mu)	O.D. at pHs (KOH)			
	11.8	12.0	12.2	12.4
400	0.080	0.085	0.080	0.100
450	0.081	0.085	0.085	0.102
500	0.145	0.150	0.155	0.160
525	0.165	0.170	0.175	0.180
550	0.175	0.178	0.182	0.184
565	0.140	0.143	0.150	0.150
575	0.130	0.132	0.140	0.140
600	0.115	0.118	0.110	0.110
625	0.080	0.070	0.065	0.060
650	0.035	0.035	0.035	0.035

(Fig. 25.)

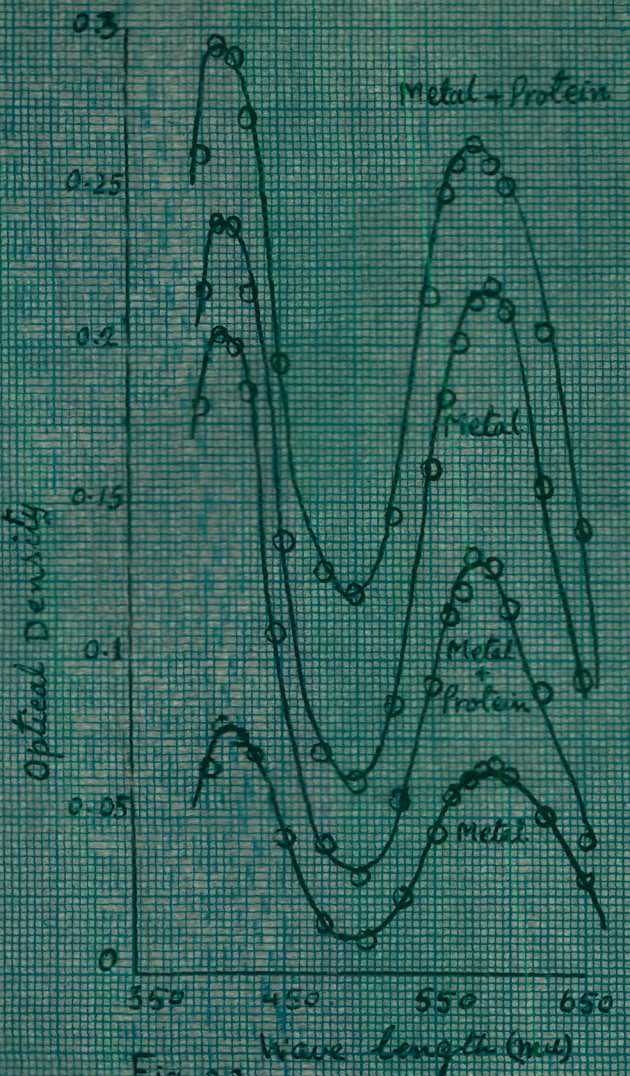


Fig 22

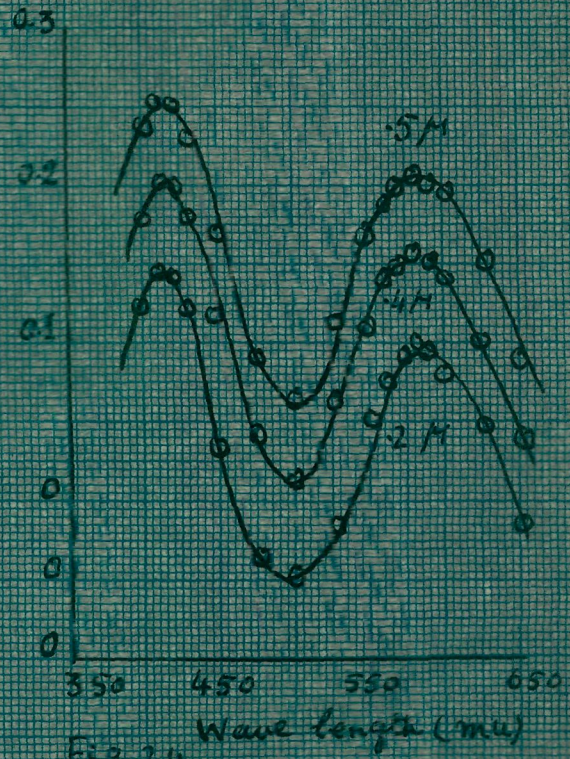


Fig 24

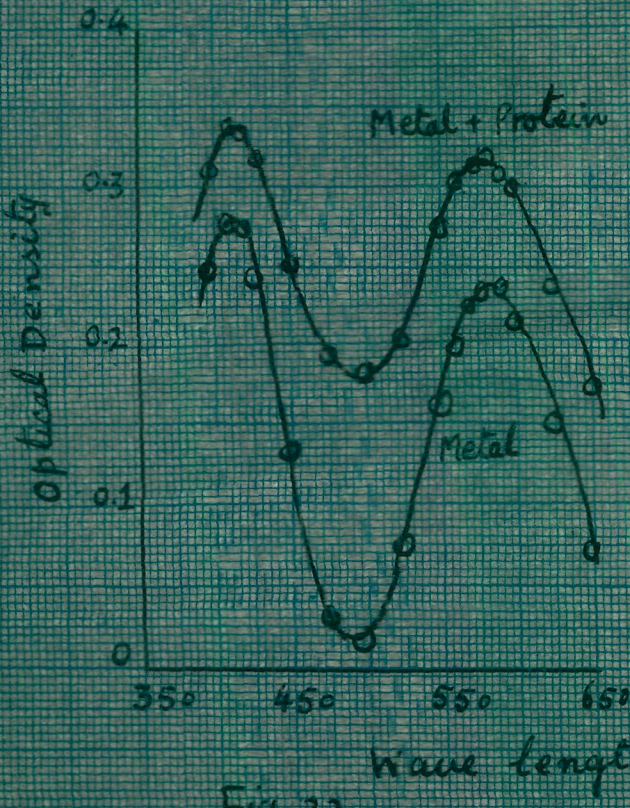
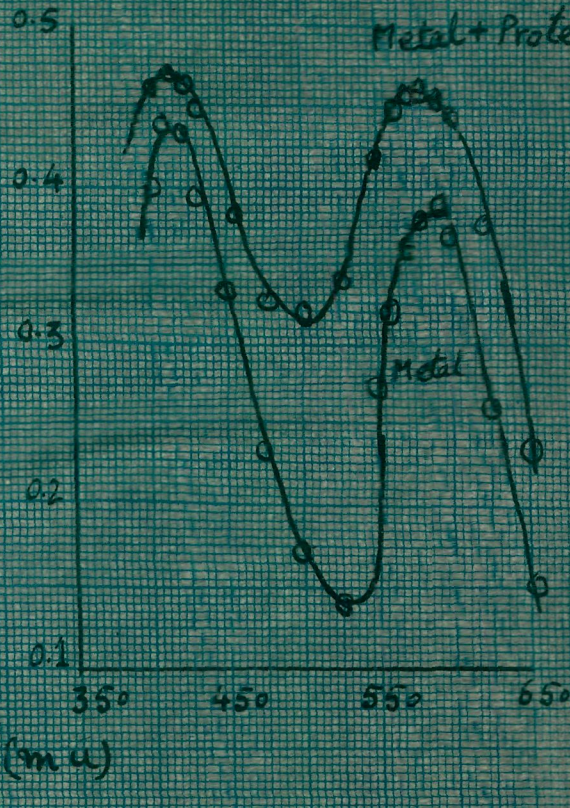


Fig 23



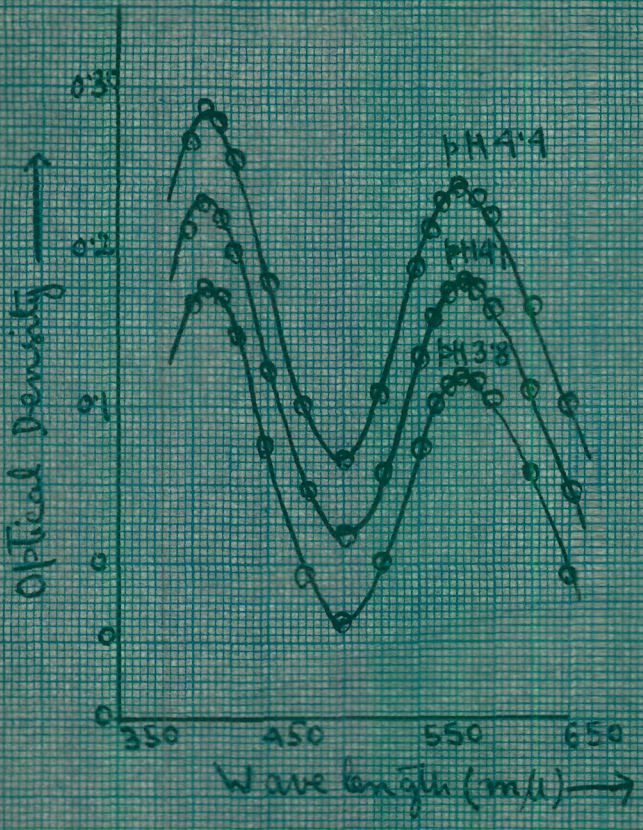


Fig. 18

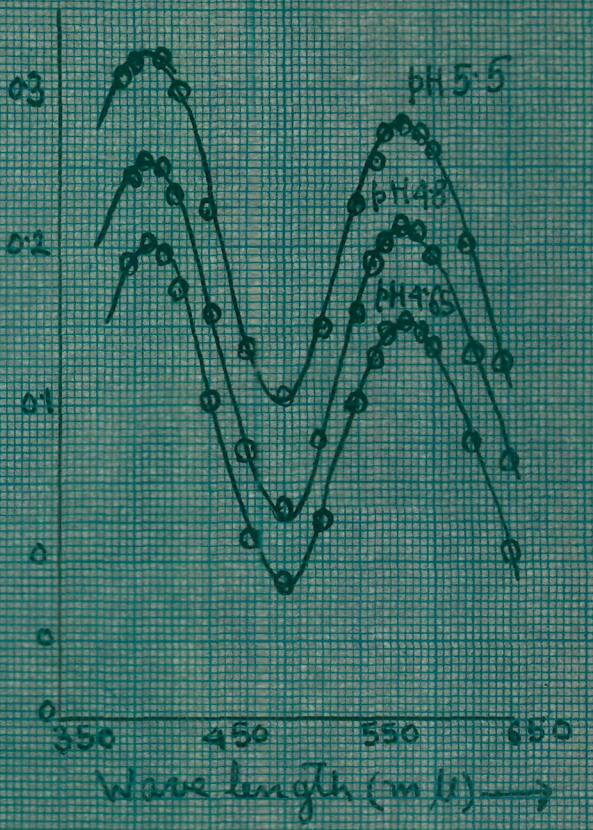


Fig. 19

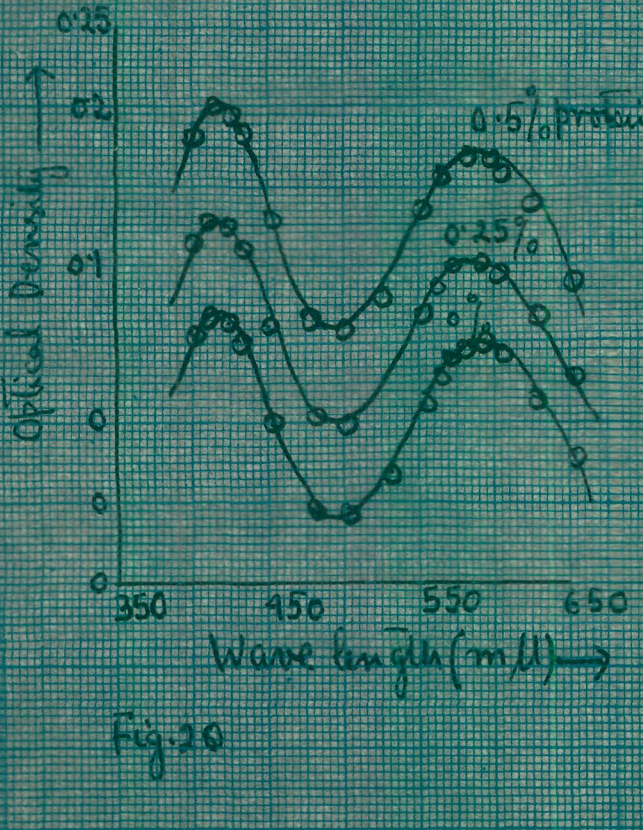


Fig. 20

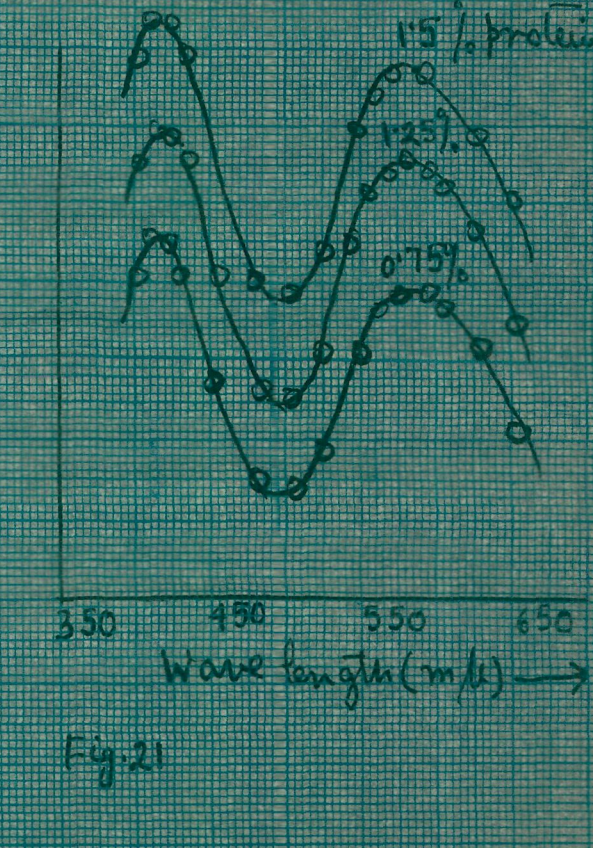


Fig. 21

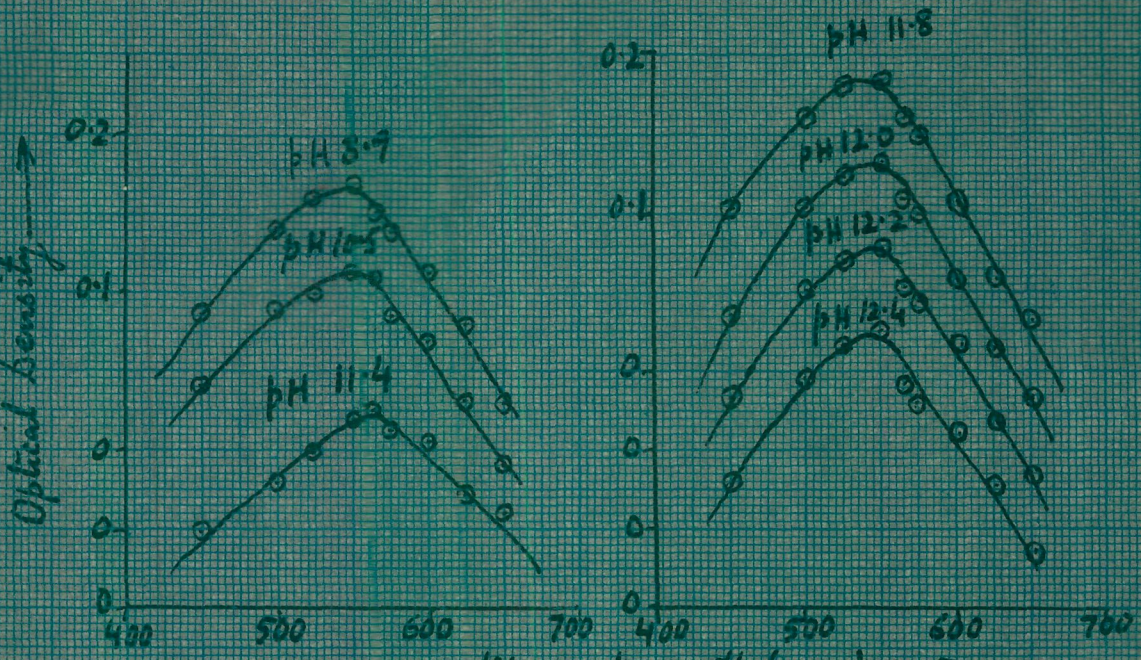


Fig 25

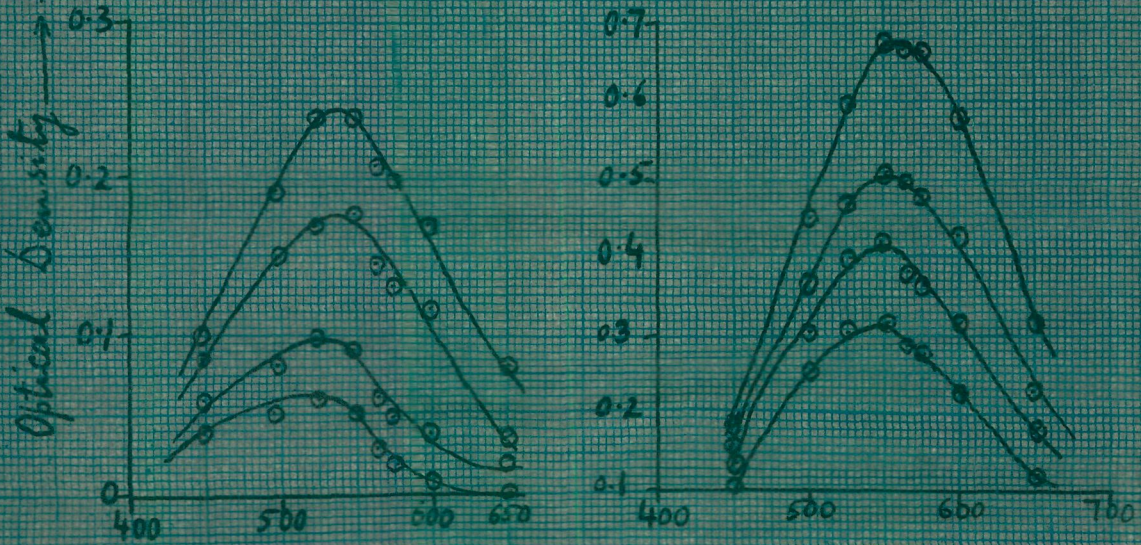


Fig 26

Curves 1, 2, 3, 4, 5, 6, 7 and 8 for 0·2, 0·5, 1·0, 1·5, 2·0, 2·5, 3·0 and $4·0 \times 10^{-3}$ M

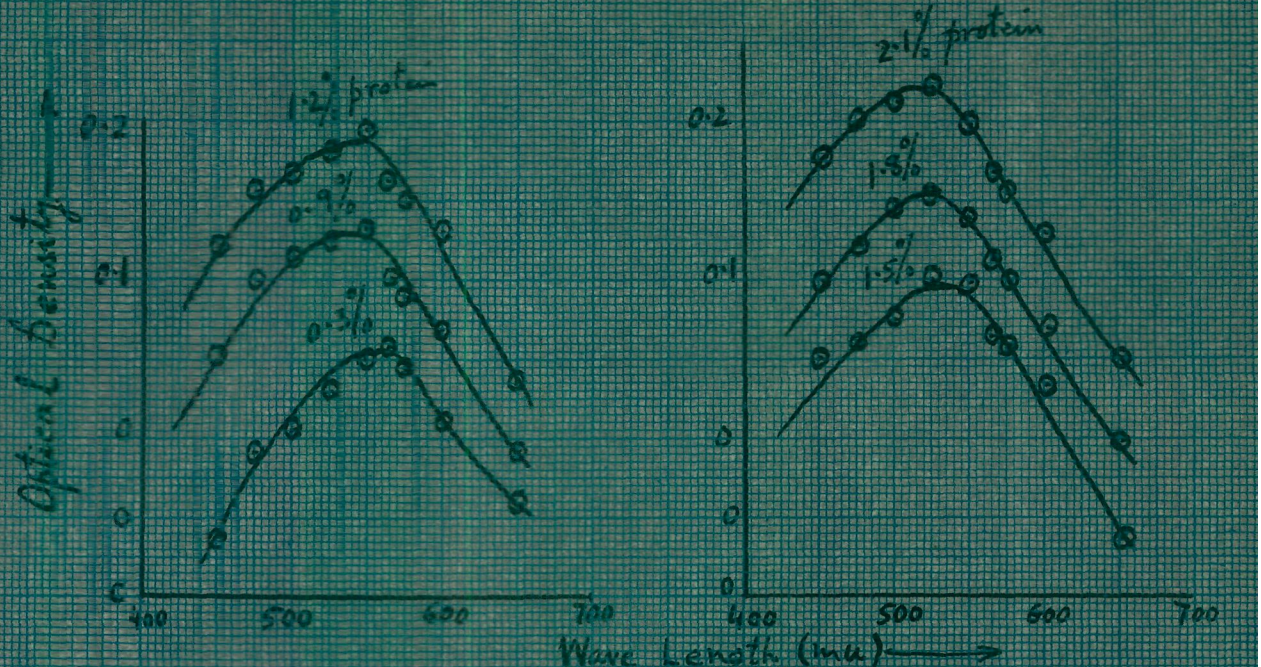

Optical density
0·2
0·1
0
400
500
600
700
1·2% protein
0·9%
0·3%
2·1% protein
1·3%
1·5%
Wave Length (mμ)

Fig. 27

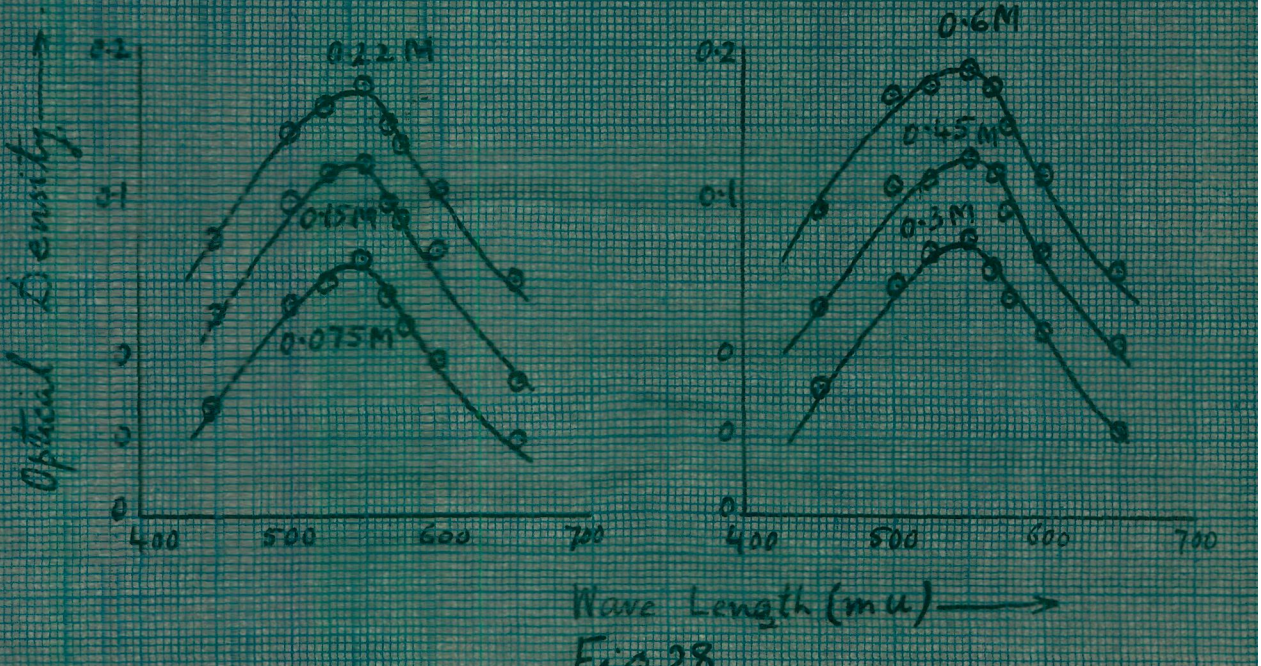

Optical Density
0·2
0·1
0
400
500
600
700
0·12 M
0·45 M
0·075 M
0·6 M
0·45 M
0·3 M
Wave Length (mμ)

Fig 28

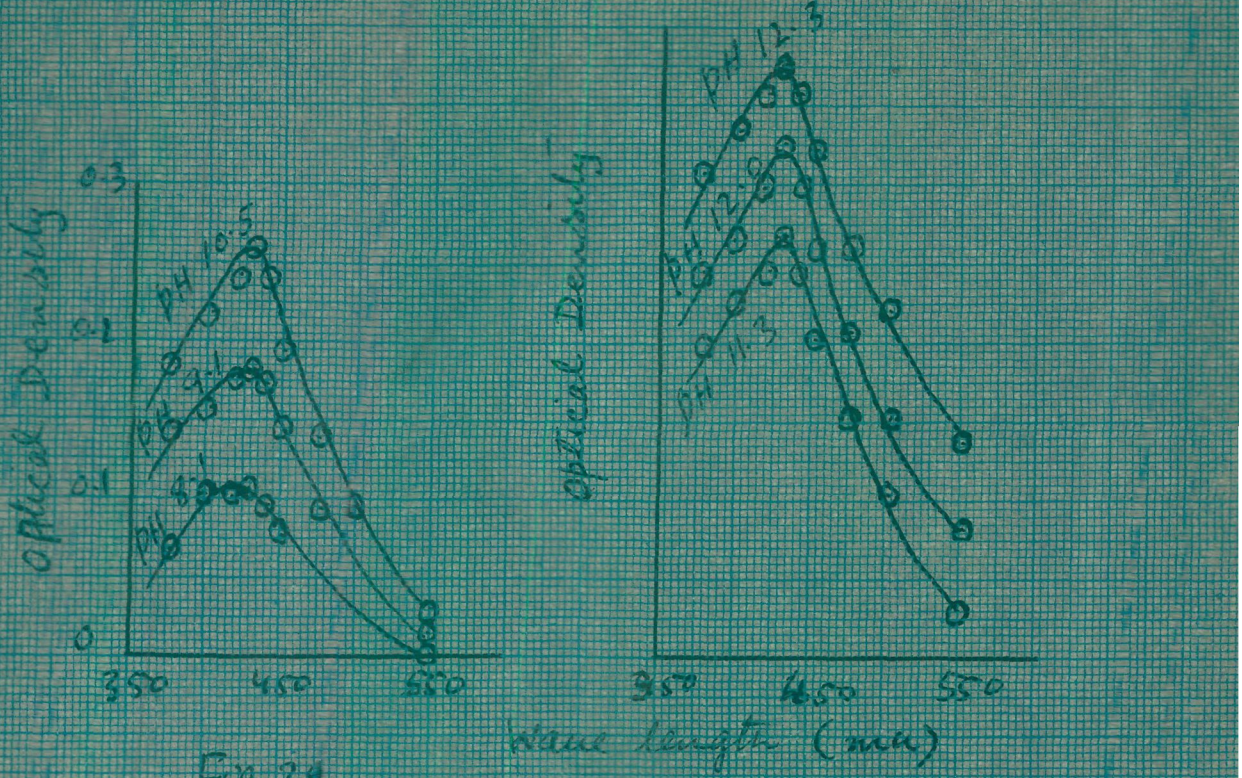


Fig 2-1

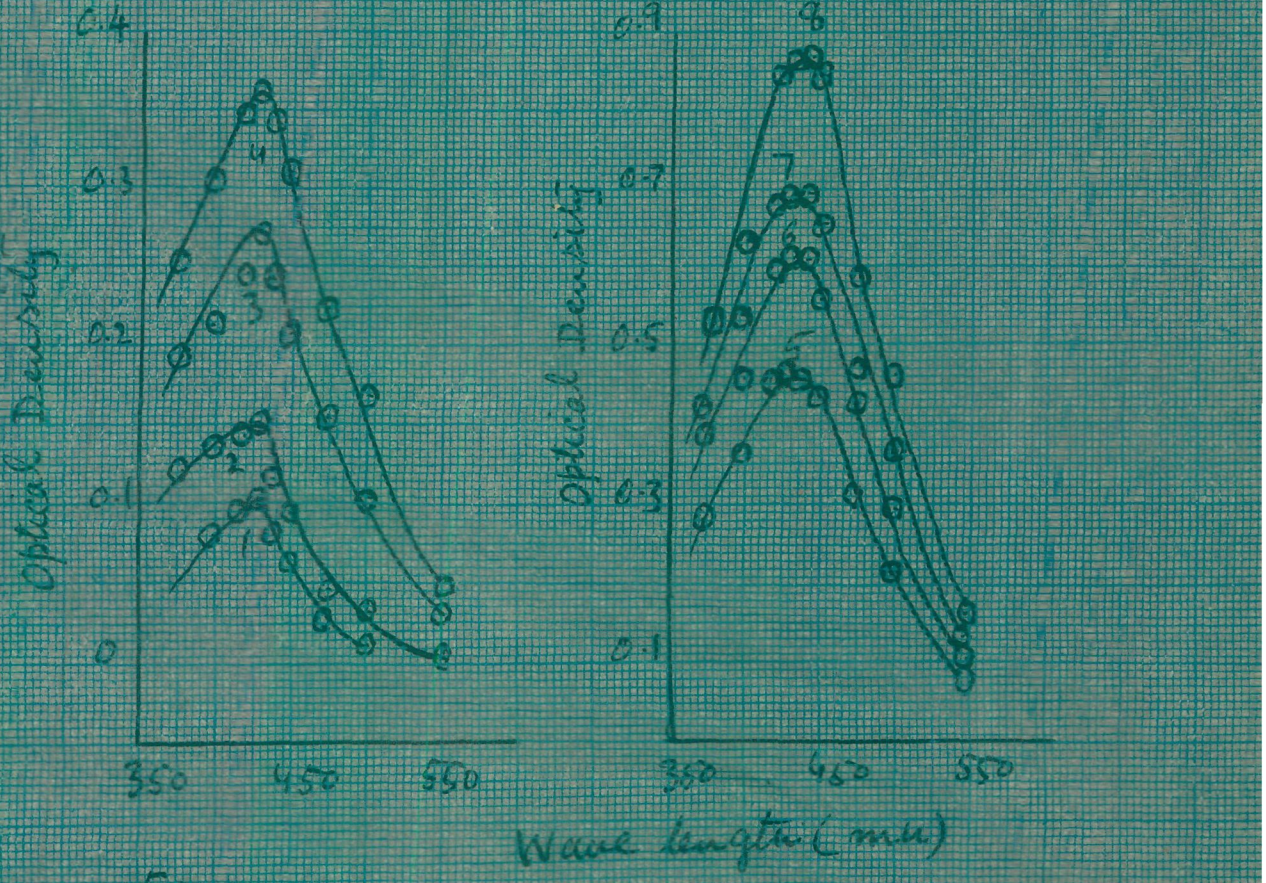


Fig 3-0

Curves 1, 2, 3, 4, 5, 6, 7 and 8 for 0.3, 0.65, 1.5, 2.25, 3.0, 3.75, 4.5 and 6.0 $\times 10^{-3}$ M Al^{+++}

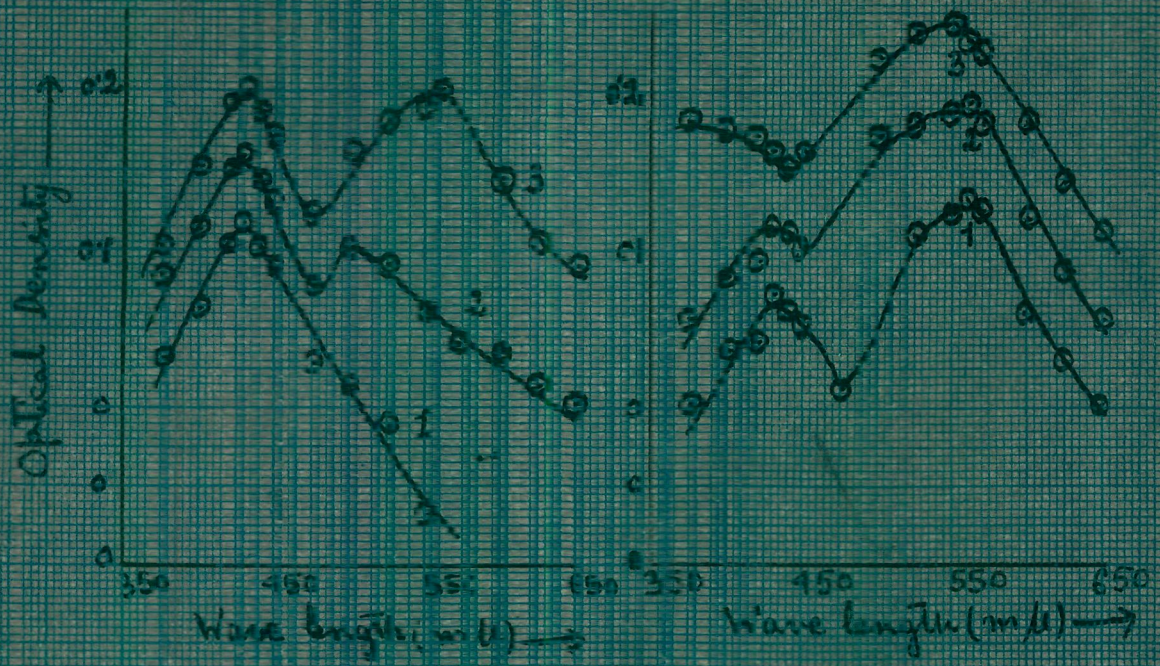


Fig: 33 Curves 1, 2 and 3 for $c = 0.25 \times 10^{-3}$ M Cu^{++} respectively

Fig: 34 Curves 1, 2 and 3 for 1.0×10^{-3} M and 2.0×10^{-3} M Cu^{++} respectively

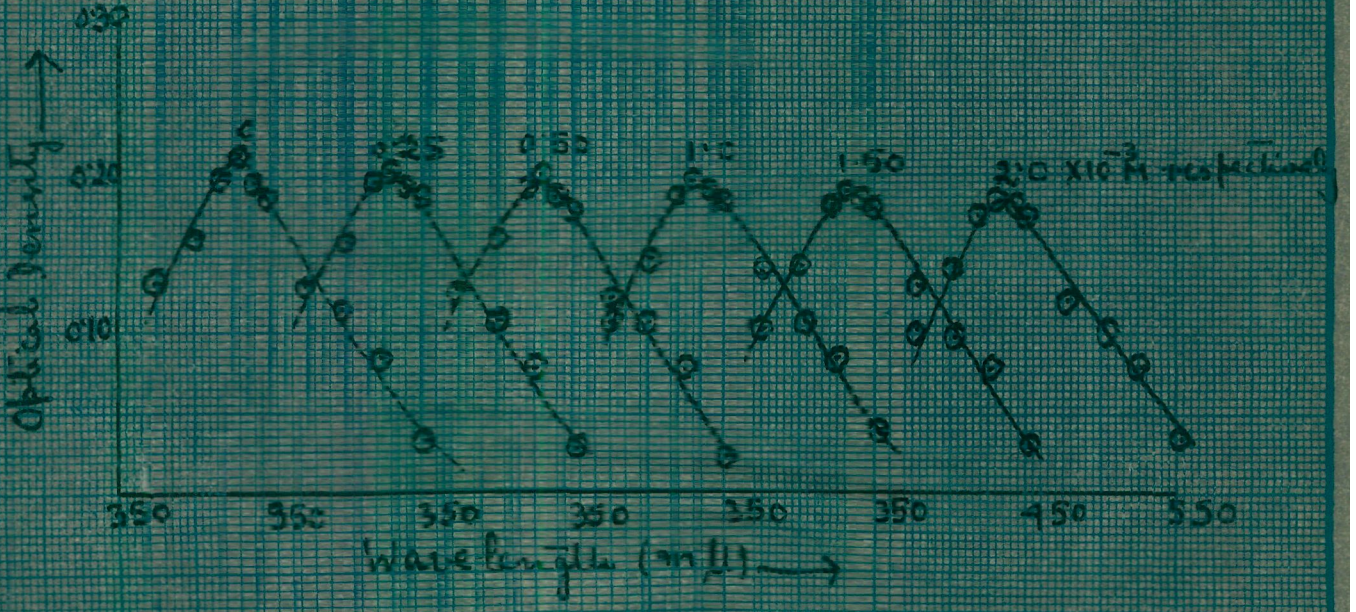


Fig 35

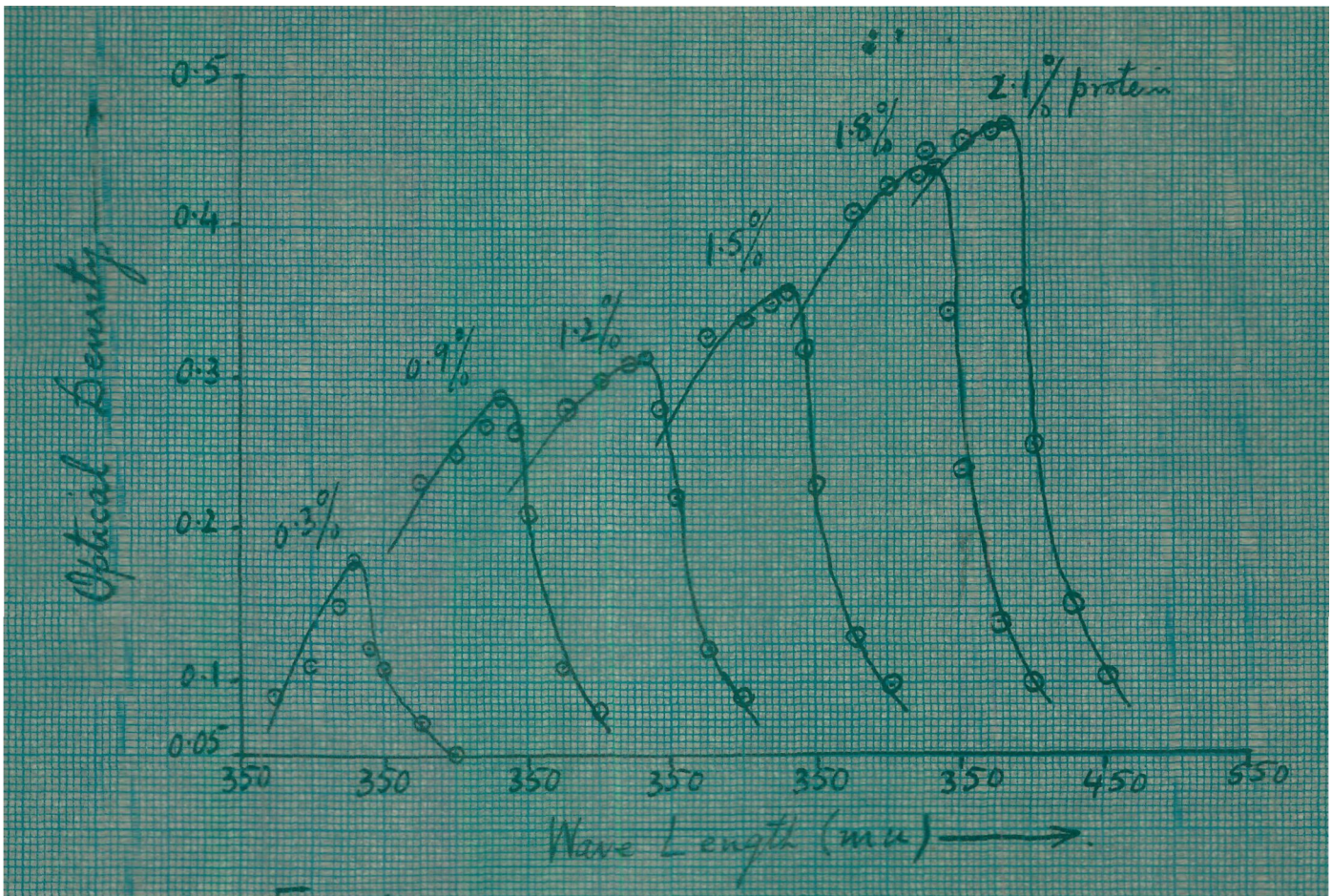

Optical Density
0·5
0·4
0·3
0·2
0·1
0·05
0·3%
0·9%
1·2%
1·5%
1·8%
2·1% protein
350
350
350
350
350
350
450
550
Wave Length (mμ)

Fig 31

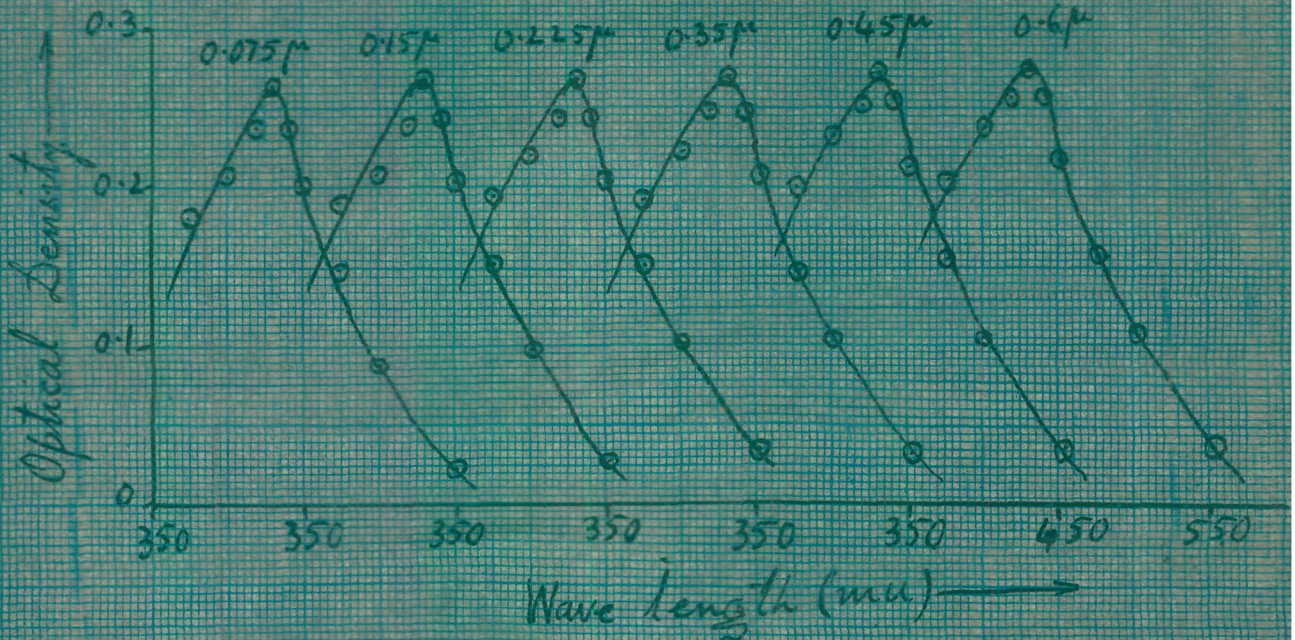

Optical Density
0·3
0·2
0·1
0
0·075μ
0·15μ
0·225μ
0·35μ
0·45μ
0·6μ
350
350
350
350
350
350
450
550
Wave length (mμ)

Fig 32

TABLE 26.

Concentration of transfusion gelatin = 0.6 %

Total volume 10 m.l. ionic strength = 0.15

pH 12.0

Wave Length (mu)	O.D. at metal concentration ($x10^{-3}M$)			
	0.2	0.5	1.0	1.5
450	0.040	0.060	0.085	0.100
500	0.050	0.080	0.150	0.190
525	0.060	0.100	0.170	0.240
550	0.050	0.090	0.178	0.240
565	0.030	0.060	0.143	0.210
575	0.020	0.050	0.132	0.200
600	0.020	0.040	0.118	0.170
650	0	0.020	0.035	0.080

Wave Length (mu)	O.D. at metal concentration ($x10^{-3}M$)			
	2.0	2.5	3.0	4.0
450	0.110	0.140	0.160	0.180
500	0.250	0.310	0.370	0.450
525	0.310	0.400	0.480	0.600
550	0.320	0.420	0.510	0.680
565	0.290	0.380	0.500	0.670
575	0.280	0.370	0.480	0.660
600	0.230	0.320	0.425	0.580
650	0.125	0.185	0.230	0.320

(Fig. 26.)

T A B L E 27.

Concentration of Cupric Chloride = 1.0×10^{-3}M.

Total volume 10 m.l. ionic strength 0.15

pH 12.0

Wave Length (mu)	O.D. at protein concentration		
	0.3 %	0.9 %	1.2 %
400	0.045	0.108	0.130
450	0.035	0.100	0.120
475	0.090	0.155	0.158
500	0.105	0.160	0.165
525	0.130	0.172	0.180
550	0.150	0.185	0.195
565	0.160	0.150	0.160
575	0.148	0.140	0.150
600	0.120	0.120	0.130
650	0.060	0.040	0.035

Wave Length (mu)	O.D. at protein concentration		
	1.5 %	1.8 %	2.1 %
400	0.170	0.200	0.240
450	0.150	0.150	0.175
475	0.160	0.170	0.200
500	0.175	0.195	0.201
525	0.200	0.205	0.220
550	0.198	0.190	0.200
565	0.165	0.160	0.162
575	0.160	0.150	0.155
600	0.135	0.120	0.130
650	0.038	0.048	0.050

(Fig. 27.)

TABLE 28.

Concentration of Cupric Chloride = 1.0×10^{-3}M

Concentration of transfusion gelatin = 0.6 %

Total volume 10 m.l. pH 12.0

Wave Length (mu)	O.D. at ionic strength		
	0.075	0.15	0.225
400	0.085	0.085	0.080
450	0.070	0.080	0.078
500	0.140	0.150	0.148
525	0.150	0.170	0.165
550	0.165	0.178	0.177
565	0.145	0.143	0.150
575	0.120	0.132	0.130
600	0.100	0.118	0.110
650	0.050	0.035	0.050

Wave Length (mu)	O.D. at ionic strength		
	0.3	0.45	0.6
400	0.081	0.100	0.160
450	0.080	0.085	0.095
500	0.150	0.160	0.170
525	0.170	0.165	0.175
550	0.179	0.180	0.185
565	0.160	0.170	0.175
575	0.135	0.140	0.150
600	0.120	0.130	0.150
650	0.055	0.060	0.070

(Fig. 28.)

TABLE 29.

Concentration of nickel Chloride = 1.5×10^{-3}M

Concentration of transfusion gelatin = 0.6 %

Total volume 10 m.l. ionic strength = 0.15

Wave Length (mu)	O.D. at pHs (KOH)			
	7.3	7.75	8.1	9.1
375	0.080	0.095	0.110	0.140
400	0.060	0.085	0.100	0.150
420	0.055	0.075	0.098	0.170
430	0.050	0.070	0.102	0.180
440	0.040	0.060	0.090	0.190
450	0.030	0.052	0.075	0.140
475	0.028	0.030	0.050	0.090
500	0	0.015	0.025	0.050
550	0	0	0	0.010

Wave Length (mu)	O.D. at pHs (KOH)			
	10.5	11.3	12.0	12.3
375	0.180	0.195	0.190	0.200
400	0.210	0.220	0.210	0.230
420	0.235	0.238	0.240	0.250
430	0.260	0.265	0.270	0.270
440	0.235	0.240	0.242	0.255
450	0.190	0.198	0.205	0.218
475	0.130	0.150	0.155	0.160
500	0.090	0.105	0.100	0.120
550	0.020	0.025	0.030	0.035

(Fig. 29)

TABLE 30.

Concentration of transfusion gelatin = 0.6 %

Total volume 10 m.l. ionic strength = 0.15

pH 12.0

Wave Length (mu)	O.D. at metal concentration ($x10^{-3}MNi^{++}$)			
	0.3	0.65	1.5	2.25
375	0.100	0.120	0.100	0.250
400	0.080	0.130	0.210	0.300
420	0.090	0.140	0.240	0.340
430	0.100	0.150	0.270	0.360
440	0.080	0.110	0.242	0.340
450	0.060	0.090	0.205	0.310
475	0.020	0.040	0.155	0.220
500	0.010	0.030	0.100	0.165
550	0	0	0.030	0.040

Wave Length (mu)	O.D. at metal concentration ($x10^{-3}MNi^{++}$)			
	3.0	3.75	4.5	6.0
375	0.290	0.380	0.410	0.520
400	0.360	0.450	0.530	0.620
420	0.450	0.590	0.680	0.850
430	0.460	0.600	0.685	0.860
440	0.470	0.610	0.690	0.865
450	0.430	0.550	0.650	0.840
475	0.310	0.420	0.460	0.580
500	0.220	0.285	0.360	0.460
550	0.070	0.100	0.120	0.145

(Fig. 30.)

TABLE 31.

Concentration of nickel chloride = 1.5×10^{-3} M

Total volume 10 m.l. ionic strength = 0.15

pH 12.0

Wave Length (mu)	O.D. at protein concentration		
	0.3 %	0.9 %	1.2 %
375	0.090	0.230	0.280
400	0.110	0.250	0.300
420	0.150	0.270	0.310
430	0.180	0.290	0.315
440	0.120	0.265	0.280
450	0.110	0.210	0.220
475	0.075	0.110	0.120
500	0.050	0.080	0.090
550	0	0.005	0.010

Wave Length (mu)	O.D. at protein concentration		
	1.5 %	1.8 %	2.1 %
375	0.330	0.410	0.450
400	0.340	0.430	0.460
420	0.350	0.435	0.465
430	0.360	0.440	0.470
440	0.320	0.325	0.338
450	0.230	0.240	0.250
475	0.130	0.140	0.150
500	0.100	0.100	0.105
550	0.020	0.030	0.030

(Fig. 31.)

TABLE 32.

Concentration of nickel Chloride = 1.5×10^{-3}M

Concentration of transfusion gelatin = 0.6 %

Total volume 10 m.l. pH 12.0

Wave Length (mu)	O.D. at ionic strength		
	0.075	0.15	0.225
375	0.185	0.190	0.195
400	0.208	0.210	0.220
420	0.240	0.240	0.245
430	0.268	0.270	0.270
440	0.240	0.245	0.245
450	0.202	0.205	0.205
475	0.150	0.155	0.155
500	0.090	0.100	0.102
550	0.025	0.030	0.035

Wave Length (mu)	O.D. at ionic strength		
	0.3	0.45	0.6
375	0.195	0.200	0.205
400	0.225	0.235	0.240
420	0.250	0.252	0.260
430	0.272	0.275	0.278
440	0.250	0.258	0.260
450	0.210	0.215	0.220
475	0.150	0.158	0.160
500	0.105	0.105	0.110
550	0.035	0.035	0.040

(Fig. 32.)

TABLE 33.

Volume of nickel + T. gelatin biuret complex = 2 m.l.

Total volume 10 m.l. ionic strength = 0.15

pH 12.0

Wave Length (mu)	O.D. at Cupric Chloride concentration ($x10^{-3}M$)		
	0	0.25	0.5
375	0.132	0.130	0.100
400	0.162	0.162	0.150
420	0.200	0.200	0.190
430	0.215	0.210	0.200
440	0.198	0.190	0.185
450	0.190	0.180	0.170
475	0.130	0.125	0.120
500	0.115	0.150	0.165
525	0.085	0.140	0.180
550	0.030	0.110	0.190
560	0.025	0.105	0.200
570	0.020	0.090	0.150
600	0	0.085	0.140
625	0	0.065	0.100
650	0	0.050	0.090

(Fig. 33)

TABLE 34.

Volume of nickel+T.gelatin biuret complex = 2 m.l.

Total volume 10 m.l. ionic strength = 0.15

pH 12.0

Wave Length (mu)	O.D. at Cupric Chloride concentration ($x10^{-3}$M)		
	1.0	1.5	2.0
375	0.100	0.102	0.180
400	0.135	0.130	0.175
420	0.140	0.140	0.170
430	0.170	0.165	0.160
440	0.160	0.160	0.150
450	0.155	0.160	0.160
475	0.110	0.220	0.315
500	0.200	0.222	0.225
525	0.215	0.225	0.235
550	0.220	0.235	0.240
560	0.230	0.240	0.230
570	0.225	0.230	0.225
600	0.160	0.170	0.180
625	0.130	0.135	0.140
650	0.100	0.105	0.110

(Fig. 34.)

TABLE 35.

Volume of nickel+T.gelatin biuret complex = 2 m.l.

Total volume 10 m.l. ionic strength = 0.15

pH 12.0

Wave Length (mu)	O.D. at Cobalt Chloride concentration ($x10^{-3}$M)		
	0	0.25	0.5
375	0.132	0.130	0.130
400	0.162	0.160	0.160
420	0.200	0.200	0.198
430	0.215	0.213	0.205
440	0.198	0.196	0.190
450	0.190	0.190	0.182
475	0.130	0.128	0.120
500	0.115	0.110	0.108
525	0.085	0.080	0.080
550	0.030	0.030	0.020

Wave Length (mu)	O.D. at Cobalt Chloride concentration ($x10^{-3}$M)		
	1.0	1.5	2.0
375	0.112	0.100	0.100
400	0.148	0.140	0.137
420	0.190	0.180	0.175
430	0.200	0.190	0.185
440	0.195	0.187	0.180
450	0.190	0.180	0.175
475	0.140	0.130	0.120
500	0.105	0.100	0.100
525	0.065	0.080	0.080
550	0.040	0.030	0.035

(Fig. 35.)

T A B L E 36.

Volume of Ni+T-gelatin biuret complex = 2 m.l.

Total volume 10 m.l. ionic strength = 0.15

pH 12.0

Wave Length (mu)	O.D. at Zinc sulphate concentration ($x10^{-3}M$)		
	0	0.25	0.5
375	0.132	0.130	0.130
400	0.162	0.160	0.155
420	0.200	0.200	0.198
430	0.215	0.215	0.210
440	0.198	0.200	0.197
450	0.190	0.195	0.190
475	0.130	0.125	0.122
500	0.115	0.100	0.100
550	0.030	0.040	0.035

Wave Length (mu)	O.D. at Zinc sulphate concentration ($x10^{-3}M$)		
	1.0	1.5	2.0
375	0.125	0.120	0.120
400	0.150	0.148	0.145
420	0.190	0.186	0.185
430	0.205	0.200	0.190
440	0.190	0.184	0.182
450	0.175	0.170	0.170
475	0.120	0.116	0.110
500	0.098	0.092	0.090
550	0.045	0.040	0.035

(Fig. 36.)

TABLE 37.

Volume of nickel+T.gelatin biuret complex = 2 m.l.

Total volume 10 m.l. ionic strength = 0.15

pH 12.0

Wave Length (mu)	O.D. at Cadmium sulphate concentration ($x10^{-3}$M)		
	0	0.25	0.5
375	0.132	0.132	0.120
400	0.162	0.150	0.145
420	0.200	0.195	0.190
430	0.215	0.214	0.210
440	0.198	0.200	0.190
450	0.190	0.190	0.180
475	0.130	0.130	0.130
500	0.115	0.120	0.110
550	0.030	0.040	0.030

Wave Length (mu)	O.D. at Cadmium sulphate concentration ($x10^{-3}$M)		
	1.0	1.5	2.0
375	0.110	0.100	0.100
400	0.135	0.120	0.118
420	0.105	0.176	0.170
430	0.205	0.200	0.190
440	0.178	0.170	0.172
450	0.170	0.160	0.160
475	0.120	0.130	0.120
500	0.115	0.110	0.100
450	0.035	0.040	0.040

(Fig. 37.)

T A B L E 38.

Volume of nickel + T.gelatin biuret complex = 2 m.l.

Total volume 10 m.l. ionic strength = 0.15

pH 12.0

Wave Length (mu)	O.D. at lead nitrate concentration ($x10^{-3}$M)		
	0	0.25	0.5
375	0.132	0.120	0.098
400	0.162	0.150	0.145
420	0.200	0.200	0.198
430	0.215	0.215	0.212
440	0.198	0.200	0.198
450	0.190	0.180	0.175
475	0.130	0.130	0.120
500	0.115	0.110	0.110
550	0.030	0.040	0.025

Wave Length (mu)	O.D. at lead nitrate concentration (x 10^{-3}M)		
	1.0	1.5	2.0
375	0.100	0.110	0.108
400	0.140	0.140	0.135
420	0.196	0.194	0.190
430	0.210	0.208	0.200
440	0.195	0.195	0.190
450	0.170	0.166	0.160
475	0.125	0.121	0.120
500	0.108	0.104	0.100
550	0.040	0.035	0.035

(Fig. 38.)

RESULTS AND DISCUSSION

The Molar Extinction coefficient E of the metal ions was calculated by means of the expression

$$\log \frac{I_o}{I} = ECd.$$

where C is the molar concentration of metal ions under study, d is the depth of the cell (1 cm for Beckman Du spectrophotometer and 1/2 inch for Bausch and Lomb spectronic 20) and $\log \frac{I_o}{I}$ is the observed optical density at the wave length of maximum absorption.

Chromium transfusion gelatin and chromium bovine serum albumin complexes absorb, maximum light at 580 mu. Therefore the extinction coefficient values for these complexes were calculated taking $\log \frac{I_o}{I}$ at 580 mu, copper biuret complex absorps maximum light in the vicinity of 550 mu, while nickel biuret complex absorbs at 430 mu. Therefore, extinction coefficient values for these complexes were calculated taking $\log \frac{I_o}{I}$ at 550 and 430 mu respectively. The results are summarised in the following tables.

TABLE 39.

Effect of pH, metal and protein concentration on Chromium transfusion gelatin interaction.

A. Protein concentration = 1.8 %

Cr(iii) concentration = 1 x 10^{-2}M

Ionic strength = 0.4

(i)	pH (with acetate buffer)	3.7	4.4	4.8	5.2	5.57	5.8
	E-values (at 580 mu)	24.2	25.4	27.7	29.0	30.0	30.4
(ii)	pH (with KOH)	3.6	4.2	4.4	4.7	4.9	5.2
	E-values (at 580 mu)	32.5	33.5	34.5	35.4	36.5	37.2

(Fig. 39 A)

B. Protein concentration 1.5 %

pH = 5.57 Ionic strength = 0.4

Chromium concentration (x 10^{-2}M)	0.25	0.50	0.75	1.0	1.5	2.0	3.0
E-values (at 580 mu)	48.8	33.0	26.8	24.0	21.0	19.8	18.1

(Fig. 39 B)

C. Cr(iii) concentration = 1 x 10^{-2}M

pH 5.57 Ionic strength = 0.4

Protein concentration (%)	0	0.6	0.9	1.2	1.5	1.8	2.1	3.5	4.0
E-values (at 580 mu)	14.8	16.2	17.7	20.8	24.0	30.4	30.8	33.0	35.2

(Fig. 39 C)

D. Effect of ionic strength on Chromium transfusion gelatin interaction

Protein concentration = 1.8 % max = 580 mu

Chromium concentration 1 x 10^{-2}M	E-values at ionic strength		
	0.5	0.4	0.2
0.5	48,0	41.2	39.6
1.0	32.0	31.6	31.5
2.5	-	18.6	18.3
3.0	20.1	20.3	20.5
4.0	19.0	19.2	19.5

TABLE 40.

Effect of pH, metal and protein concentration on the Chromium-bovine serum albumin interaction.

A. Protein concentration = 1.0 %

Cr(iii) concentration = 1 x 10^{-2}M Ionic strength = 0.15

(i) pH (with acetate buffer)	3.7	4.4	4.8	5.2	5.5	5.9	6.3
E-values (at 580 mu)	15.2	15.8	18.3	19.8	21.6	22.6	23.0

(ii) pH (with KOH)	3.6	4.1	4.4	4.65	4.8	5.15
E-values (at 580 mu)	22.0	22.5	23.5	24.8	25.9	27.0

(Fig. 40 A)

B. Protein concentration = 1.0 %

pH = 5.5 Ionic strength = 0.15

Chromium concentration (x 10^{-2}M)	0.5	1.0	1.5	2.0	3.0
E-values (at 580 mu)	29.0	21.6	18.1	16.4	15.8

(Fig. 40 B)

C. Cr(iii) concentration = 1.0×10^{-2}M

pH = 5.5 Ionic strength = 0.15

Protein concentration (%)	0	0.25	0.5	0.75	1.25	1.5
E-values (at 580 mu)	14.8	15.6	17.4	19.8	22.6	23.2

(Fig. 40 C)

Effect of ionic strength on Chromium bovine serum albumin interaction

Protein concentration = 1.0 %

Cr(iii) Concentration = 1.0×10^{-2}M

pH = 5.5

Ionic strength	0.15	0.2	0.4	0.5
E-values (at 580 mu)	21.6	22.0	22.6	22.8

TABLE 41.

Effect of pH, metal and protein concentration and ionic strength on copper transfusion gelatin interaction

A. Concentration of cupric chloride = 1.0×10^{-3}M

Concentration of transfusion gelatin = 0.6 %

Ionic strength = 0.15

pH	max. (mu)	O.D.	E-values
5.4	-	-	-
6.8	625	0.095	76.0
8.9	565	0.125	100.0
10.5	550	0.165	132.0
11.4	550	0.170	136.0
11.8	550	0.175	140.0
12.0	550	0.178	142.0
12.2	550	0.182	145.0
12.4	550	0.184	147.2

B. Concentration of transfusion gelatin = 0.6

pH = 12.0 Ionic strength = 0.15

Metal concentration $\times 10^{-3}$M	max. (mu)	O.D.	E-values
0.2	525	0.060	240.1
0.5	525	0.100	163.7
1.0	550	0.178	142.4
1.5	550	0.240	128.0
2.0	550	0.320	128.0
2.5	550	0.420	134.4
3.0	550	0.510	136.0
4.0	550	0.680	136.0

C. Concentration of cupric chloride = 1.0×10^{-3}M

pH = 12.0 Ionic strength = 0.15

Protein concentration (%)	max. (mu)	O.D.	E-values
0.3	565	0.160	128.0
0.6	550	0.178	142.4
0.9	550	0.185	148.0
1.2	550	0.195	156.0
1.5	525	0.200	160.0
1.8	525	0.205	164.0
2.1	525	0.220	176.0

D. Concentration of cupric chloride = 1.0×10^{-3}M

Concentration of transfusion gelatin = 0.6 %

pH = 12.0

Ionic strength ($\Gamma/2$)	max. (mu)	O.D.	E-values
0.075	550	0.165	132.0
0.150	550	0.178	142.4
0.225	550	0.177	141.6
0.300	550	0.179	143.2
0.450	550	0.180	144.0
0.600	550	0.185	148.0

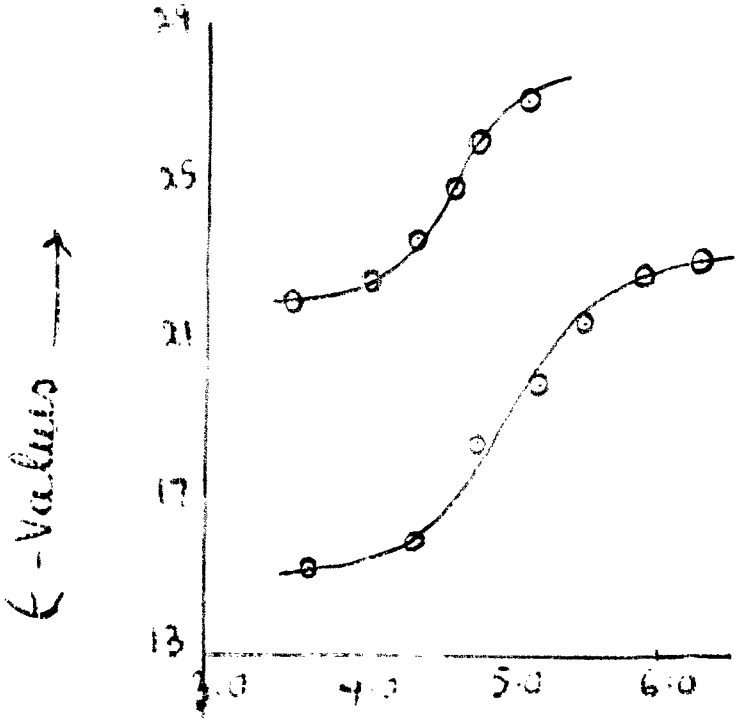


Fig. 40 A

Effect of pH on Cr - bovine serum albumin interaction.

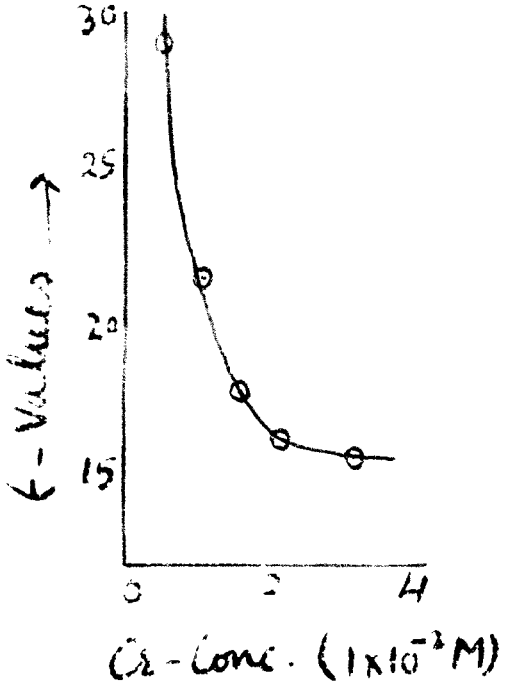


Fig. 40 B

Effect of metal ion Conc. on Cr - bovine serum albumin interaction

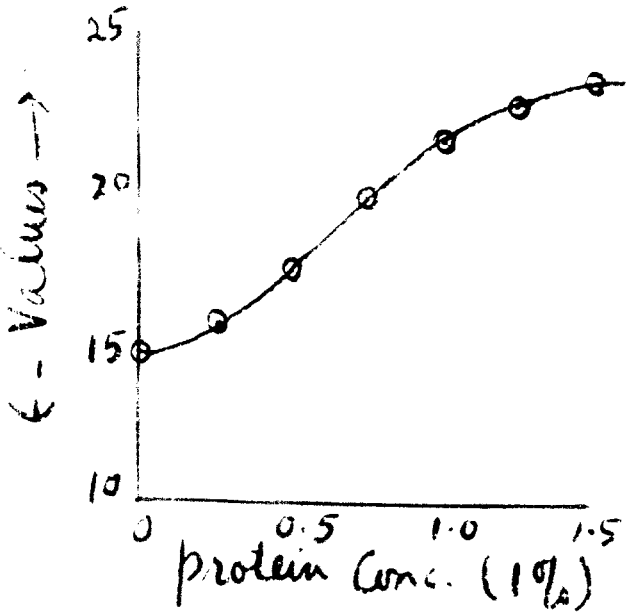


Fig 40 C

Effect of protein Conc. on Cr - bovine serum albumin interaction.

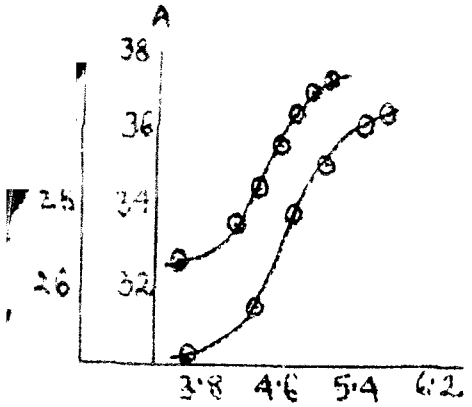


Fig 39A:
Effect of pH on Cr–transfusion gelatin interaction

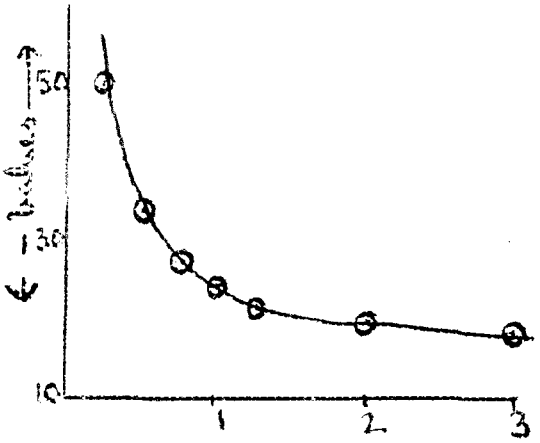


Fig. 39B:
Effect of metal ion – Concentration on Cr–transfusion gelatin interaction

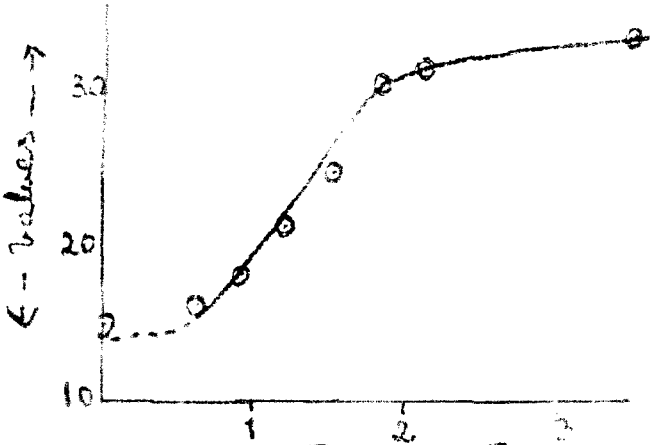
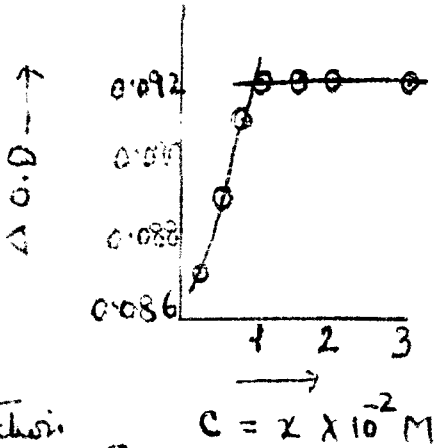


Fig: 39C Effect of protein concentration on Cr–transfusion gelatin interaction



C = x X 10^{-2} M

Fig 39D:
Plot of Δ O.D (difference in optical densities of Chromium–gelatin mixture and that of Chromium alone) against C (Chromium – Concentration).

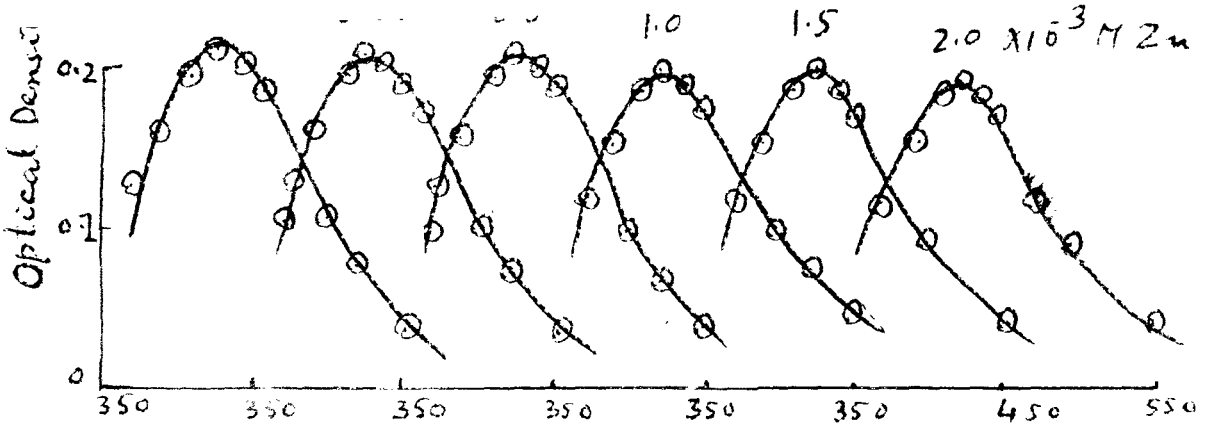


FIG-36

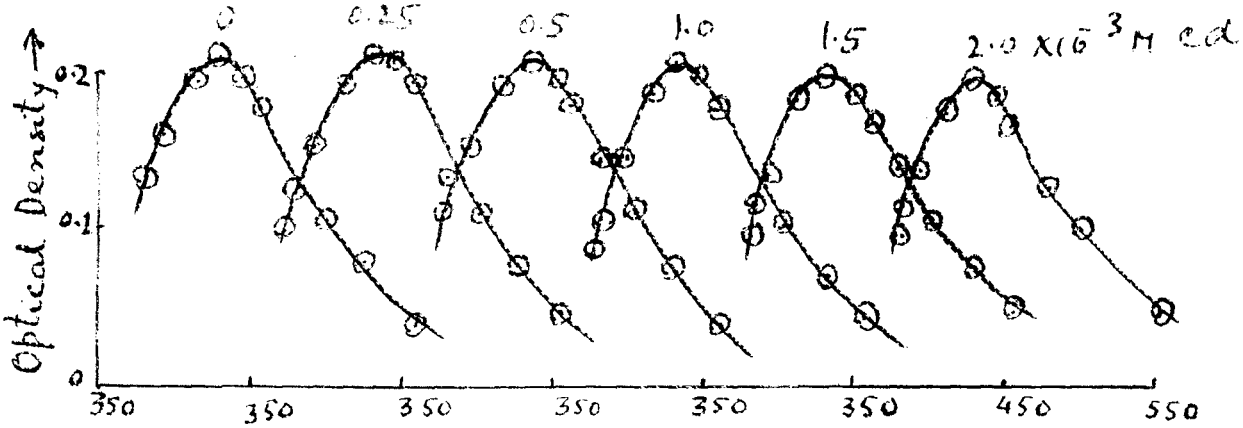


FIG-37

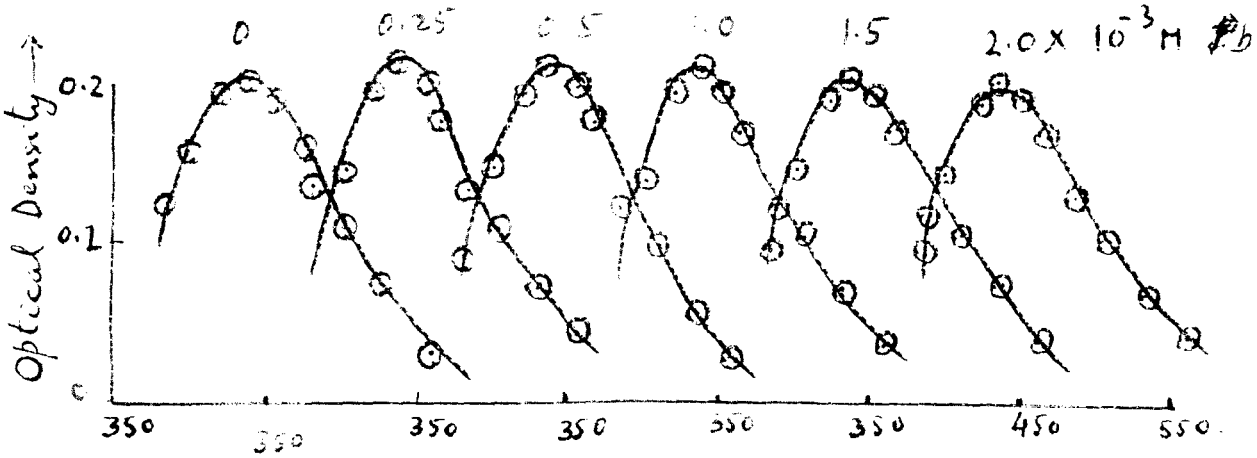


FIG:38.

TABLE 42.

Effect of pH, metal and protein concentration, and ionic strength, on the extent of nickel transfusion gelatin interaction

A. Concentration of nickel chloride = 1.5×10^{-3}M

Concentration of transfusion gelatin = 0.6 %

Ionic strength = 0.15

pH	max (mu)	O.D.	E- values
7.30	-	-	-
7.75	-	-	-
8.10	430	0.102	54.4
9.10	430	0.180	96.0
10.50	430	0.260	138.7
11.30	430	0.265	141.4
12.00	430	0.270	144.0
12.30	430	0.270	144.0

B. Concentration of transfusion gelatin = 0.6 %

pH = 12.0 Ionic strength = 0.15

Metal concentration $\times 10^{-3}$M	max. (mu)	O.D.	E-values
0.30	430	0.100	266.7
0.65	430	0.150	184.6
1.50	430	0.270	144.0
2.25	430	0.360	128.0
3.00	430	0.460	122.7
3.75	430	0.600	128.0
4.50	430	0.685	121.8
6.00	430	0.860	114.7

TABLE 42. (Contd.)

C. Concentration of nickel chloride = 1.5×10^{-3}M

pH = 12.0 Ionic strength = 0.15

Protein concentration (%)	max. (mu)	O.D.	E- values
0.3	430	0.180	96.0
0.6	430	0.270	144.0
0.9	430	0.290	154.6
1.2	430	0.315	165.4
1.5	430	0.360	191.5
1.8	430	0.440	234.7
2.1	430	0.470	256.4

D. Concentration of nickel chloride = 1.5×10^{-3}M

Concentration of transfusion gelatin = 0.6 %

pH = 12.0

Ionic Strength (Γ/2)	max. (mu)	O.D.	E-values
0.075	430	0.268	142.9
0.150	430	0.270	144.0
0.225	430	0.270	144.0
0.300	430	0.272	145.1
0.450	430	0.275	146.7
0.600	430	0.278	148.3

TABLE 43.

Effect of bivalent metal ions on the absorption spectrum of nickel transfusion complex.

2 m.l. of complex diluted to 10 m.l.

pH = 12.0, ionic strength = 0.15

(Total concentration of Ni^{++} = 1.0×10^{-3}M)

max. 430 mu

Concentration of metal ions ($\times 10^{-3}$M)	O.D. of the complex in presence of				
	Cu^{++}	Co^{++}	Zn^{++}	Cd^{++}	Pb^{++}
0	0.215	0.215	0.215	0.215	0.215
0.25	0.210	0.213	0.215	0.214	0.215
0.50	0.200	0.205	0.210	0.210	0.212
1.00	0.170	0.200	0.205	0.205	0.210
1.50	0.165	0.190	0.200	0.200	0.208
2.00	0.160	0.185	0.190	0.190	0.200

It is a well known fact that protein molecules are capable of binding metal cations. These metal ions are bound with the aid of oxygen, nitrogen, and sulphure atoms located in the periphery of the protein molecule. The site of oxygen is that of ionised carboxyl groups, phenolic groups, alcoholic hydroxyl groups or peptide carbonyl groups. Nitrogen is furnished by amino groups, the acid amide and from peptide linkage. Sulphur containing groups are those ionised sulphahydryl groups, thioether and disulphide bond. Since the state of bound

metal ions essentially differs from that in the aquous solution, therefore a change in the optical property should be accompanied with complex formation. Moreover, the absorption spectra of a coloured substance (particularly of coloured metal ions), often change, when the substance form complexes with simple ligand, and so the resultent spectra would be a characteristics of the nature of linkage. Similarly it has been found that, in the presence of suitable protein, the absorption spectra of metal ions are modified, and the shifts which proteins produce in the spectra of coloured metal ions, may be compared with those caused by simple organic ligands containing known functional groups. Hence valuable information could be made available regarding the nature of bond by this method.

Combination of Cr(iii) with transfusion gelatin and bovine serum albumin

The absorption studies carried out in the pH range 3.7 to 5.8 at different wave lengths give a maximum at 580 mu, showing thereby the binding of the metal ions through the carboxyl groups[33] of transfusion gelatin and bovine serum albumin. Moreover, since no shift in maximum is observed even at pH 5.8, it may be concluded that imidazole groups of the proteins are not involved in binding chromic ions (expected λ max. 540 mu Green and Ang[31] on Cr(iii) alanine complex, when

donar nitrogen atoms are involved in complex formatiom).

The molar extinction coefficient values at 580 mu when plotted against pH give an S-shapped curve. The inflexion occuring near about pH 5.0. This result again indicates that a large number of carboxyl groups are made available in this pH range (84 groups per transfusion gelatin molecule[34] and 100 groups per bovine serum albumin molecule[35]) and offer the maximum number of sites for the binding of chromium. A comparison of extinction coefficient values (for both the protein) measured in acetate buffers and KOH also give a few interesting features of the interaction process. It is found that E-values are larger in KOH medium than in the acetate buffers. From these results it may be concluded that the acetate ions exert a negative influence on the metal protein interaction. These results may be interpreted in the light of Gustavson's[36] assumption who visualises a lesser reactivity of chromium ions in presence of the acetate solution due to the formation of uncharged chloro-species $(Cr_2(OH)_5Cl)^o$ of the chromic ions.

Metal protein ratio appears to exert a large influence on the binding of chromium to the proteins. It is evident, from the results on the spectrophotometric titrations carried out with metal ions in presence of a fixed amount of protein and vice versa; that the presence of a large proportion of metal ions in the reaction mixture brings about a decrease in the binding capacity. On the other hand, presence of larger amounts of protein in the

reaction mixtures invariably brings about an increase in the binding of the metal ions to the protein. On plotting the difference of optical densities of metal ion and the complex [illegible] against metal concentration, an inflexion, in case of Cr(iii) transfusion gelatin system, is observed at metal concentration equal to 0.85×10^{-2}M. This interesting observation leads to conclude that the maximum binding takes place at the metal protein ratio $0.85 \times 10^{-2} : 0.2 \times 10^{-3}$ that is 42 : 1. In case of Cr(iii) bovine serum albumin no such regular curve is obtained.

Experiments performed at three ionic strengths, viz., 0.2, 0.4 and 0.5 go to show that the extent of metal protein interaction increases with increase in ionic strength. The effect is most pronounced in dilute solutions rather than in concentrated one, where almost no change in extinction coefficient values is observed with increase in the ionic strength (table 39 D).

One of the interesting features of the chromic ion binding to both the proteins is that the transfusion gelatin has got greater binding capacity as compared to bovine serum albumin (the molar extinction coefficient of chromic ions is greatly increased in presence of transfusion gelatin than with bovine serum albumin). The great reactivity of transfusion gelatin itself reveals the structural difference between fibrous and globular proteins. The peptide chains of fibrous proteins

being more or less extended and oriented in parallel pattern[37], may form a intra-or intermicellar cross linking. This type of linkage, as demostrated by Gustavson[37] may contain several chromium atoms, by means of two or more carboxyl groups of adjacent protein chains. In fact Kuntzel[38] have shown on the basis of birefringence studies, that, intramicellarly combination occured between collagen and vegetable tannin. On the other hand globular proteins have a compact and folded structure and such a multipoint combination may not be possible in case of serum albumin. Hence, low binding capacity of bovine serum albumin, may be attributed to the lack of such effective multipoint sites so as to fix several chromium atoms intramicellarly.

The Biuret Reaction of transfusion gelatin

The absortion studies carried out in a wide pH range (5.4 to 12.4 for copper transfusion gelatin and 7.3 to 12.3 for nickel transfusion gelatin systems) at different wave lengths give a maximum at 550 mu for copper and at 430 mu for nickel complexes, showing thereby the binding of these metal ions to the peptide nitrogen atom (the characteristic peak for nickel biuret complex is 433 mu and 565 mu for copper biuret complex[39]).

Metal protein ratio appears to exert a profound influence on the binding of copper and nickel to the

protein. From the results (table 41 and 42) it may be concluded that the presence of a large proportion of metal ions in the reaction mixture brings about a decrease in the binding capacity. On the other hand, the presence of larger amounts of protein invariably brings about an increase in the binding of metal ions (as evident from the increase in molar extinction coefficient values). The marked difference between the two systems, viz., copper-transfusion gelatin and nickel-transfusion gelatin, is that cupric ions form two types of complexes namely red as well as violet. At lower protein concentrations (metal ions fixed) a violet complex is formed, which absorbs maximum light at 550 mu, while at higher protein concentration a red complex is formed and absorption spectra are shifted towards the shorter wave length (525 mu) with a subsequent increase in extinction coefficient values. On the other hand nickel forms only one complex in the entire range of protein concentration, These observation leads one to conclude that under the biuret condition, peptide oxygen may also be involved in binding cupric ions. Hence the complex is bound to show a shift in absorption maximum.

One of the interesting features of the present investigation is that, a number of bivalent metal cations, which have been added in increasing amount to the nickel transfusion gelatin complex, show the characteristic effect on absorption spectrum of the complex under consideration. The gradual addition of copper caused progres-

sively dimunition of the absorption peak so much so that at high copper concentration, the absorption spectrum of nickel-transfusion gelatin completely disappears, and a new absorption spectrum appears at 550 mu. This observation clearly shows the greater affinity of copper for peptide nitrogen (or oxygen) than for nickel. The amount with which, the different bivalent metal ions reduce the absorption peak (430 mu) may be taken as a measure of the relative affinities for these metal ions to the peptide nitrogen. The values listed in table (43) reveal the following increasing order for the metal ions studied.

$$Pb < Zn = Cd < Co < Ni < Cu.$$

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P A R T III.

Studies on the mutual interaction of metals and their hydrous oxide sols with casein and egg albumin by pH-metric and viscosity methods.

I N T R O D U C T I O N

Numerous references on the mutual interaction of hydrophobic sols are available in the existing literature. Amongst the important contributors are Billitzar[1], Lottermoser[2], Blitz[2], Thomas[4], Nathansohn[5], Thomas and Johnson[6] Weiser and Chapman[7] and Hazel and McQueen[8], Weiser and Chapman[7] suggested the following factors influencing the mutual coagulation: (i) adsorption of colloidal particles, (ii) the presence of precipitating ions as impurities in the sol and (iii) interaction between stabilising ions. Hazel and McQueen[8] also pointed out that the mutual adsorption of oppositely charged sols would result in the unequal distribution of charges around the particles, with a consequent coagulation of the two sols. Weiser and Milligan[9] also have shown that mutual coagulation was accompanied by lowering of zeta potential of the two sols and the displacement of their counter ions. Studies on the mutual coagulation of sols are of some significance especially in the mechanism of coagulation of negatively changed sols by cations such as chromium and aluminium, which are partially hydrolysed in aqueous solutions.

Mixtures of colloidal hydrophobic and hydrophilic solutions offer results of still greater interest. Usually the effect is the protection of hydrophobic sol either

through purely physical effects or due to complex formation between the two interacting sols. On the other hand Freundlich[10,11], Pauli[12,13] and Andrieve[14] observed that the unstable hydrophobic sols were stabilised in excess amount of protein, where as small quantities of protein sensitise or even coagulate the sol. The sensitisation of gold sol (negatively charged) by acidified gelatin may be explained in terms of flocculation whereas the sensitisation of the same sol above pH[15] 7 may be due to the formation of agglomerates of gold sol. The agglomerates are formed due to partial covering of the hydrophobic colloid by gelatin, while protection is affected by the complete covering. The thickness of the protected layer of gelatin on gold sol was found to be of the order of 10^{o}A (monolayer)[16]. The idea of the protection of hydrophobic particles due to multilayer adsorption of protecting agent was, however rejected by William and Chang[17]. Jirgensons[18] and Poeter and Matalon[19] demonstrated that the protecting power of the hydrophilic substances some what depended upon its structure. He observed that the protective action was due to the adsorption of gelatin on the surface of the sol particles through the undissociated carboxyl groups. The principle underlying protection has been utilised in preparing hydrophobic sols of high degree of dispersion, which are widely used in medicine.

Recently, Bull and coworkers[20-22] investigated

the problem of the interaction of cationic proteins with negatively charged glass particles. These authors observed that the electrophoretic mobility of the protein molecule depended upon the charge, the surface of the glass particles, and the pH of the solution. According to them mutual interaction was affected through the partial neutralisation of the negative charge on the glass particle by means of the positive protein molecule. However, they did not take into account the role of possible ionising groups of the protein.

Undoubtedly much work has been done on the interaction of hydrophobic and hydrophilic colloids from the purely physical view point. Very few references are available in which the attempt is made to interpret the results in the light of the colloidal-chemical properties, associated with hydrophilic system. The study should therefore become fascinating when the hydrophilic colloid is of a protein, with its large number of reactive groups made available at various pH-values. Pauli[13] for the first time, drew the attention to the chemical aspect of the problem, during the course of his studies on the mutual interaction of albumin and congo blue. He observed that equal concentration of two sols resulted in their mutual coagulation, where as the greater concentration of protein caused a change in colour from blue to red. The possible explanation offered by Pauli was that complex formation between the nitrogen atom (NH_3^-) of the dye and the carboxyl group of the protein. Another

work worth mentioning in this connection is that of Kvyat[23], who carried out extensive studies on the effect of a number of organic substances including amino acids and protein on the formation of hydrous oxide sols of iron and aluminium. He found that unlike other organic substances, benzoic and salicylic acids (carboxyl group) protected the hydrophobic sols at a smaller concentration (0.002M) and sensitised at higher concentration. Besides this, he observed marked changes in the structural viscosity during the interaction of the two sols with egg albumin, peptone and alanine.

More recently comprehensive studies on this problem have been undertaken by Malik and co-workers.[24-27] Considering the protein as a multivalent electrolyte and the availability of the various reactive groups at different pH-values for interaction, these authors treated the problem in the light of possible combination of metal ions, with the protein from the electrolyte, as well as from the inner part of the electrical double layer of their hydrous oxide sols. In view of the far-reaching importance of such work especially from the biochemical view point, it was thought worthwhile to extend these studies, to other protein like egg albumin, casein etc. The results of the viscometric and pH-metric studies on the mutual interaction of metals and their hydrous oxide sols with egg albumin and casein are described in this part of the thesis.

pH-metric method

Attention to the importance of this method in investigating the metal protein complexes[28] have been drawn in earlier chapter (Chapter 2 part 1). The underlying principle of the method may be summarised as follows:

Metal salt solutions and their hydrous oxide sols were titrated with anionic proteins. The displacement in the titration curves when the protein is replaced by alkali of the same pH and electrolyte, or sol with acid solution of the same pH, is utilised to ascertain the mode of combination between the metal and the protein.

Viscometric method

The factors influencing the viscosity of colloidal solutions are, charge, size and shape of the particles.[29] The variation in viscosity of sols by the addition of electrolyte or during their mutual coagulation may be attributed to any of these factors, or their combined effect. In this connection reference may be made of the work of Neurath and Putman[30], Boyer and Co-workers[31] and Kuyat[23]. The viscosity measurements have also been used to indicate structural changes during metal-protein interactions. P. Doty[32] used viscometric results supplemented by light scattering and sedimentation

studies to indicate the dimerization of serum albumin in mercuric-serum albumin solution.

EXPERIMENTAL

Apparatus and Technique

pH-measurements were carried out by means of Backman Model G. pH-meter using glass electrode. Nitrogen (purified by passing through pyrogallol and chromous chloride) was passed through the reaction mixture to ensure an inert atmosphere. All measurements were carried out at 30 $\pm$ 0.1°C, keeping the cell in water thromstate (Townson and Mercer Ltd. Coryodon).

Viscometric measurements were carried out by means of modified Scarpa's method. The method first reported by Scarpa's[33] facilitates the calculation of viscosity of solution without the knowledge of densities. The method was further improved by Farrow[34], Prasad Mehta and Desai[35].

The viscometric constant, K, (for the Ostwald type viscometer) was calculated by determining experimentally, t, the time of rise and t_2 the time of fall for a given volume of solution under constant pressure, for the liquid of known viscosity (in the present case conductivity water). The viscosity of the solution is related to t, and t_2 under constant pressure, by means of expression.

$$= \frac{K\ t_1\ t_2}{t_1 - t_2}$$

The viscosity of the test solutions were determind by finding t_1 and t_2 for a unknown volume of solution The measurements were carried out at 30 ± 0.1°C keeping the viscometer in a water thermostate (Townson and Mercer Ltd. Corydon).

Solutions and reagents

Colloidal Solutions

Alumina sol was prepared according to Weiser[36], and the concentration of colloidal solution was determined gravimetrically[37] as Al_2O_3. The pH of the sol was adjusted to 2.0. For the preparation of ferric oxide and chromic oxide sol, the methods recommended by Krecke[38] and Grahms[39], were used respectively their pH-values were adjusted to 2.0. The iron content was determined volumetrically by potassium permangnate method, and chromium content was found out colorimetrically[40] by oxidising with sodium peroxide and comparing the optical density at 373 mu with that of standard chromate solutions. Cobalt hydroxide and nickel hydroxide sols were prepared by excessive washing[41] of the corresponding hydroxide precipitate obtained by Cobalt chloride, nickel chloride with caustic soda, their strengths determined gravimetrically.[42,43]

E. Merck samples of ferric chloride, aluminium chloride, chromic chloride, cobalt chloride and nickel

chloride were used as source of metal ions. Their solutions were prepared by dissolving requisite amount of each in doubly distilled water (distilled in all glass apparatus) and concentration determined by usual method. The pH of these solutions were adjusted to desired value.

Dilute solution of carbonate free potassium hydroxide (pH 12.0) was prepared according to Kolthoff. Hydrochloric acid solution was made from A.R. quality of the acid and its pH was kept at 2.0.

The charge on the colloidal particles was determined by means of Burton's type electrophoretic tube. All the hydrous oxide sols prepared by the above method were found to be positively charged.

Protein Solutions

Cystalline egg albumin (E. Merck) was dissolved by weight in dilute alkali. The resultant solution was filtered off and pH was adjusted at 12.0, its concentration was found out by estimating nitrogen by Kjeldahl method 2.5 % by weight). Casein solution was prepared by soaking the E. Merck sample with distilled water over night, then the mixture was stirred mechanically by the addition of small amounts of dil alkali solution at a time, and the string was continued with frequent addition of alkali till the solution became clear. It was filtered off and concentration was determined by Kjeldahl method.

The pH of Casein (2.8% by weight) solution was also brought to 12.0.

Procedure

Varying volumes of viz., 0, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0 and 16.0 m.l. of hydrous-oxide or electrolyte solution were mixed with 4 m.l. of egg albumin in different pyrex boiling tubes, and the total volume was made upto 20 m.l. Such sets were prepared taking iron aluminium, chromium, cobalt, nickel and their hydrous oxide sols and egg albumin.(fixed volume 4 m.l.). The pH and viscosity of all the mixtures were determined. For viscosity measurement, those sets were selected in which coagulation or precipitation did not set in. Similar sets for all the metal ions and their hydrous oxide sols were studied, replacing egg albumin by potassium hydroxide of the same pH.

Mixtures of iron, aluminium, chromium and their hydrous oxides with casein were also studied under strictly identical conditions. The results are summarised in the following tables.

n_m and n_s are the viscosities of the mixtures (containing sol and protein), and that of the solution (containing only protein) respectively; $\frac{n_m - n_s}{n_s}$ is the change in specific viscosity.

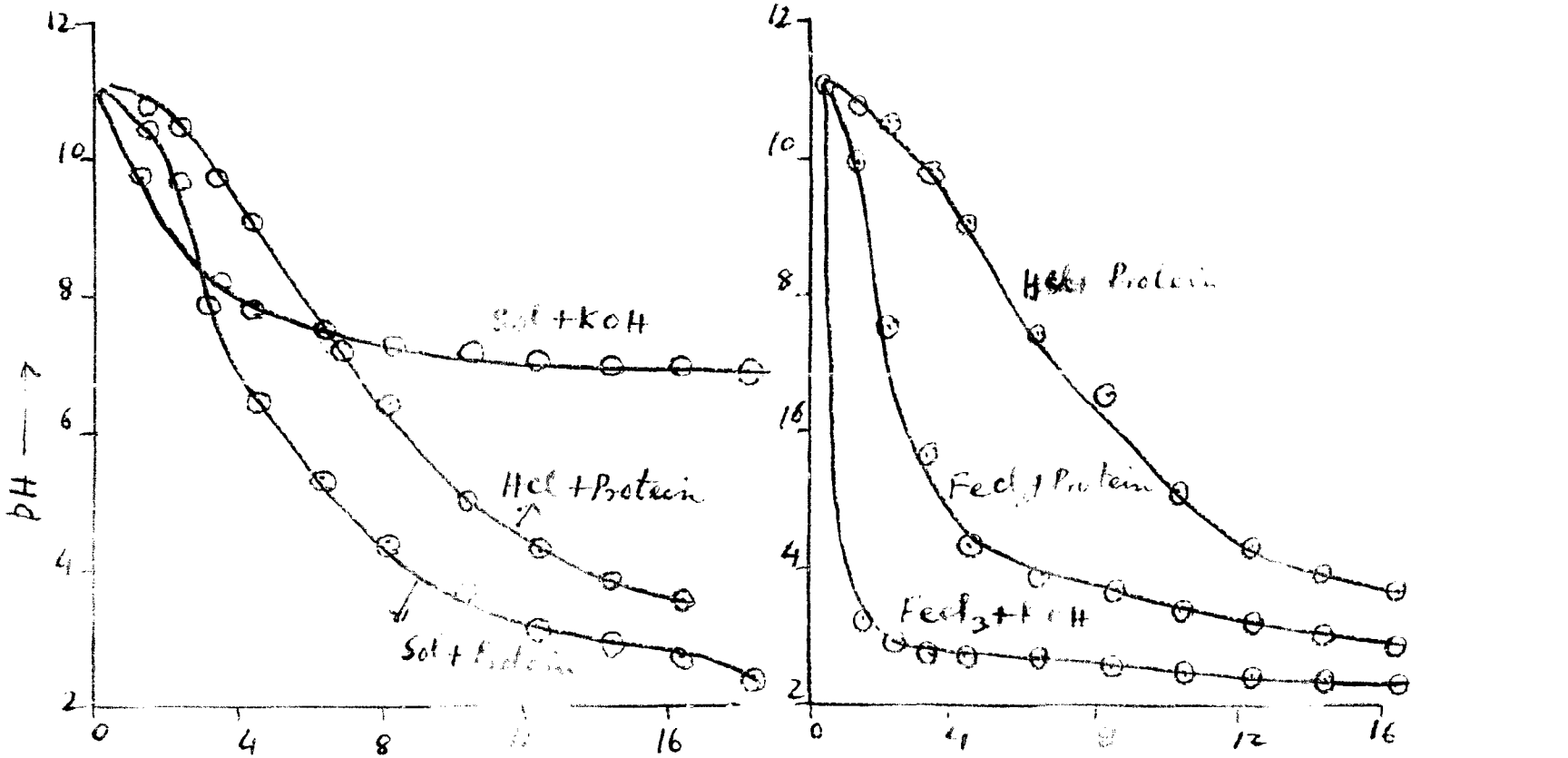
12
10
8
6
4
2
pH →
0
4
8
16
Sol + KOH
HCl + Protein
Sol + Protein
12
10
8
16
4
2
0
4
12
16
HCl + Protein
FeCl3 + Protein
FeCl3 + KOH
Volume of sol. electrolyte or HCl added (ml) →

TABLE 1.

Egg albumin solution, concentration (2.5%), pH 12.0

Ferric oxide sol, concentration (5.814 gm/L.), pH 2.0

Ferric chloride solution, concentration (2.92 gm/L.)pH 2.0

Potassium hydroxide solution pH 12.0

total volume 20 m.l. Temperature 30°C

Volume of sol or electrolyte added	Ferric oxide sol		Ferric Chloride	
	pH with egg albumin	pH with KOH	pH with egg albumin	pH with KOH
0	11.00	11.00	11.00	11.00
1	10.45	9.7	9.96	3.20
2	9.62	8.7	7.50	2.90
3	7.98	8.2	5.70	2.75
4	6.40	7.8	4.22	2.70
6	5.35	7.4	3.80	2.62
8.	4.40	7.2	3.62	2.58
10	3.55	7.1	3.25	2.45
12	3.10	7.0	3.10	2.35
14	2.88	6.9	2.95	2.30
16	2.68	6.9	2.80	2.25

(Fig. 1)

N.B. Coagulation during the mutual interaction of two sols in the vicinity of pH 6.5. No coagulation on mixing the sol or electrolyte with alkali.

TABLE 2.

Egg albumin solution, concentration (2.5%), pH 12.0

Aluminium oxide sol, concentration (8.582 gm./L.),pH 2.0

Aluminium Chloride solution, concentration(2.12gm/L)pH 2.0

Potassium hydroxide solution pH 12.0

Total volume 20 m.l. Temperature 30°C

Volume of sol or electrolyte added	Aluminium oxide sol		Almminium Chloride	
	pH with eff albumin	pH with KOH	pH with egg albumin	pH with KOH
0	11.00	11.10	11.00	11.10
1	10.40	5.00	5.00	4.50
2	9.95	3.00	4.80	4.00
3	9.50	2.85	4.50	3.70
4	9.00	2.65	4.15	3.60
6	7.40	2.55	4.05	3.40
8	4.60	2.45	3.95	3.05
10	3.70	2.40	3.80	2.90
12	3.30	2.35	3.15	2.56
14	3.10	2.30	2.85	2.35
16	2.75	2.25	2.25	2.15

(Fig. 2)

N.B. The sol was precipitated at pH 3.6 by anionic egg albumin, and metal ions were precipitated by alkali beyond pH 4.5.

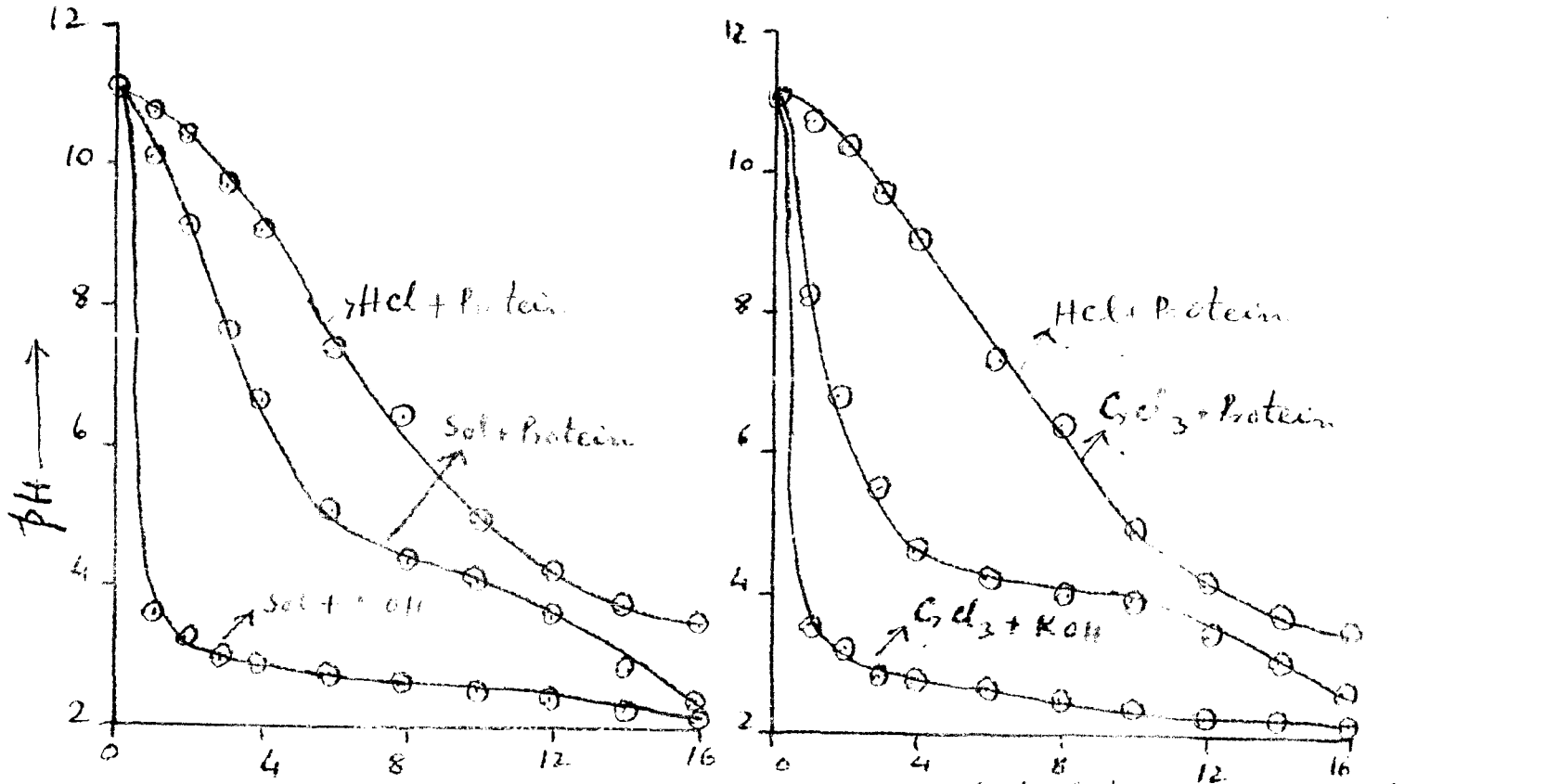
12
10
8
6
4
2
pH
0
4
8
12
16
HCl + Protein
Sol + Protein
Sol + KOH
HCl + Protein
$CrCl_3$ + Protein
$CrCl_3$ + KOH
Volume of sol, electrolyte or HCl added (ml)

FIG-3

TABLE 3.

Egg albumin solution concentration (2.5%), pH 12.0

Chromic oxide sol, concentration (0.945 gm/L), pH 2.0

Chromic chloride solution, concentration (0.657 gm/L.)pH 2.0

Potassium hydroxide solution pH 12.0

Total volume 20 m.l. Temperature 30°C.

Volume of sol or electrolyte added	Chromic Oxide sol		Chromic Chloride	
	pH with egg albumin	pH with KOH	pH with egg albumin	pH with KOH
0	11.00	11.00	11.00	11.00
1	10.10	3.62	8.20	3.50
2	9.20	3.28	6.82	3.20
3	7.60	2.98	5.52	2.95
4	6.70	2.82	4.60	2.75
6	5.10	2.75	4.30	2.70
8	4.40	2.65	4.00	2.60
10	4.10	2.50	3.90	2.42
12	3.62	2.40	3.40	2.32
14	2.85	2.29	3.10	2.24
16.	2.38	2.20	2.70	2.15

(Fig. 3)

N.B. Sol as well as metal ions were precipitated in the vicinity of pH 6.0 by anionic egg albumin and alkali.

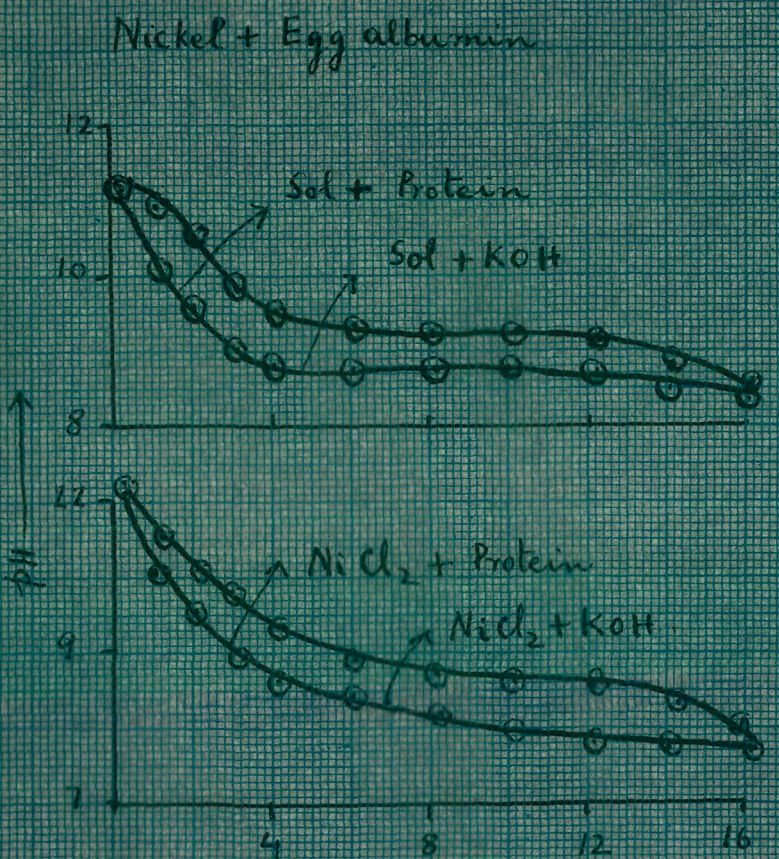

FIG-4 Vol of Sol or electrolyte added (ml) →

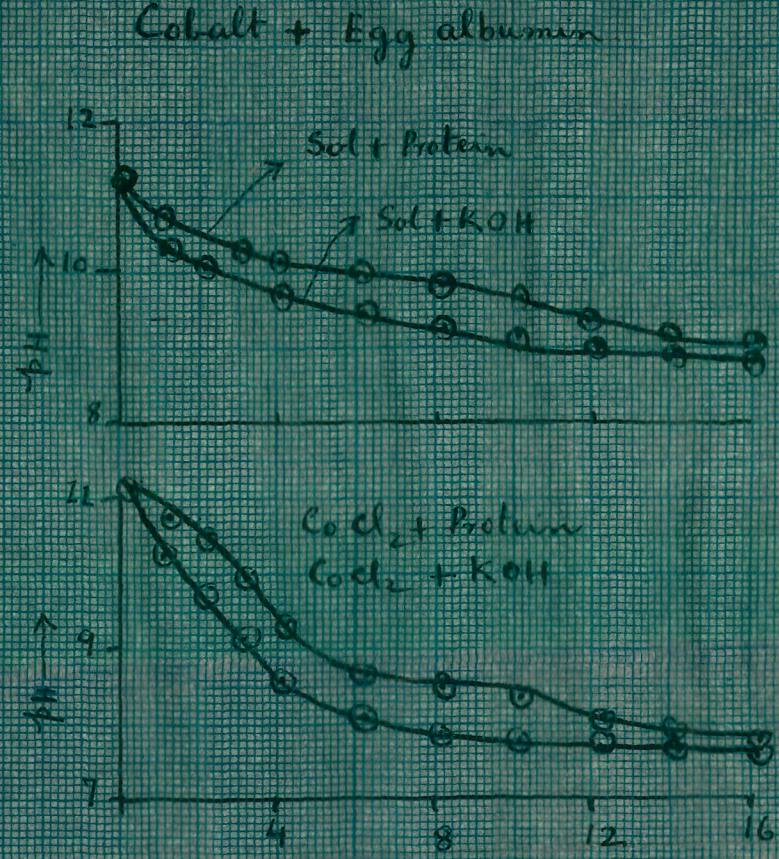

FIG-5 Vol of Sol or electrolyte added (ml) →

TABLE 6.

Egg albumin solution, concentration (2.5%), pH 12.0

Cobalt oxide sol, concentration (0.821 gm/L.), pH 7.4

Cobalt Chloride solution, concentration (2.85×10^{-2} M), pH 7.4

Potassium hydroxide solution pH 12.0

Total volume 20 m.l. Temperature 30°C

Volume of sol or electrolyte added	Cobalt oxide sol		Cobalt Chloride	
	pH with egg albumin	pH with KOH	pH with egg albumin	pH with KOH
0	11.10	11.10	11.10	11.10
1	10.60	10.20	10.70	10.20
2	10.35	10.00	10.40	9.20
3	10.20	9.50	9.90	9.10
4	10.00	9.30	9.25	8.50
6	9.85	9.15	8.60	8.00
8	9.70	9.05	8.50	7.85
10	9.60	8.95	8.40	7.72
12	9.20	8.80	7.90	7.64
14	9.15	8.80	7.80	7.60
16	9.10	8.80	7.60	7.55

(Fig. 5)

N.B. Cobalt ions were precipitated by alkali (in electrolyte as well as sol) above pH 8.0 and remained uneffected with anionic egg albumin.

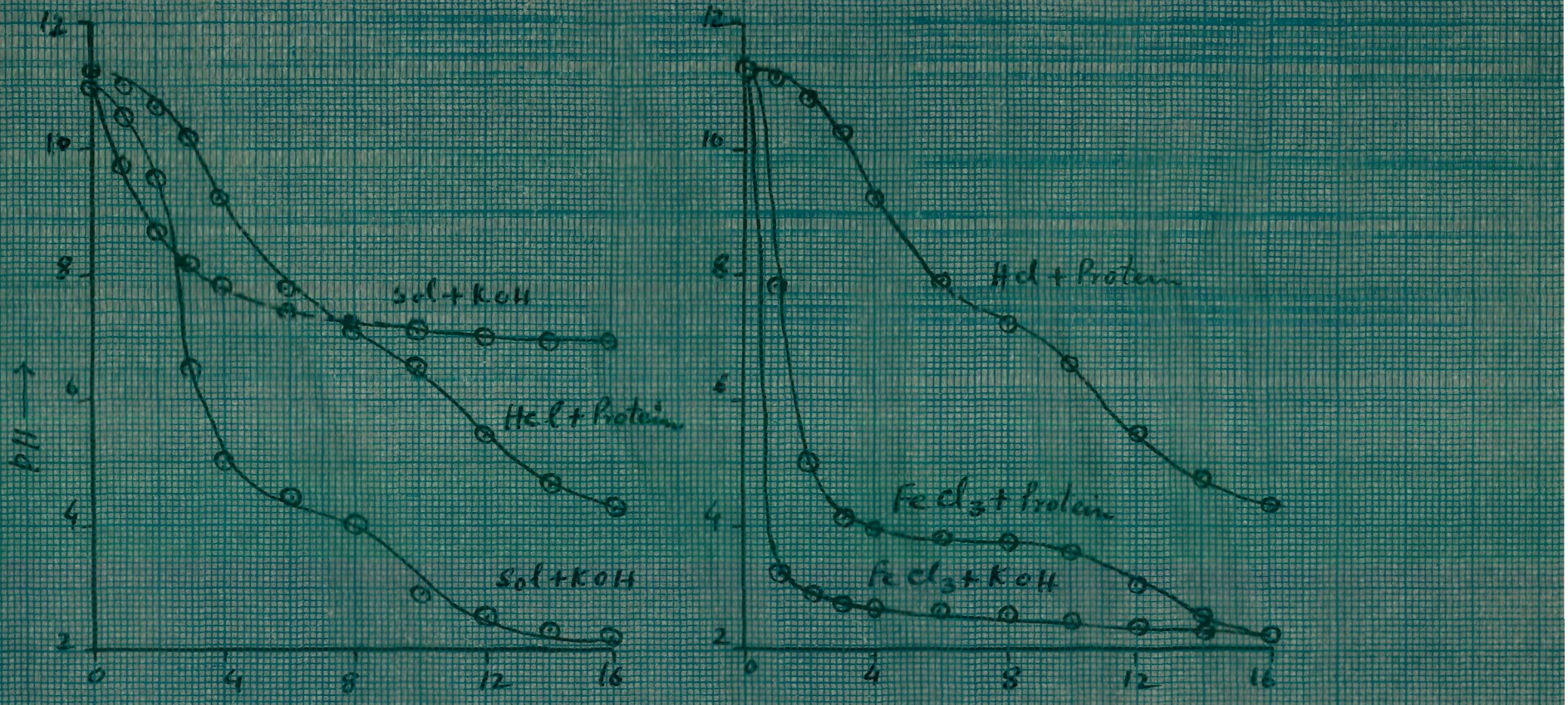
12
10
8
6
4
2
pH
0
4
8
12
16
Sol+KOH
HCl+Protein
Sol+KOH
HCl+Protein
FeCl3+Protein
FeCl3+KOH
Volume of sol, electrolyte or HCl added (ml)

FIG-6.

TABLE 7.

Casein solution, concentration (2.8%), pH 12.0

Ferricoxide sol, concentration (5.814 gm/L.), pH 2.0

Ferric Chloride solution, concentration (2.92 gm/L.),pH 2.0

Potassium hydroxide solution pH 12.0

Total volume 20 m.l. Temperature 30°C

Volume of sol or electrolyte added	Ferric oxide sol		Ferric Chloride	
	pH with Casein	pH with KOH	pH with Casein	pH with KOH
0	11.00	11.00	11.00	11.00
1	10.50	9.70	7.80	3.20
2	7.40	8.70	5.10	2.90
3	6.50	8.20	4.10	2.75
4	5.00	7.80	3.95	2.70
6	4.50	7.40	3.80	2.62
8	4.00	7.20	3.70	2.58
10	2.90	7.10	3.50	2.45
12	2.50	7.00	3.00	2.35
14	2.38	6.90	2.50	2.30
16	2.35	6.90	2.20	2.20

(Fig. 6)

N.B. Ferric oxide sol as well as ferric ions were precipitated by anionic casein at about 5 pH.

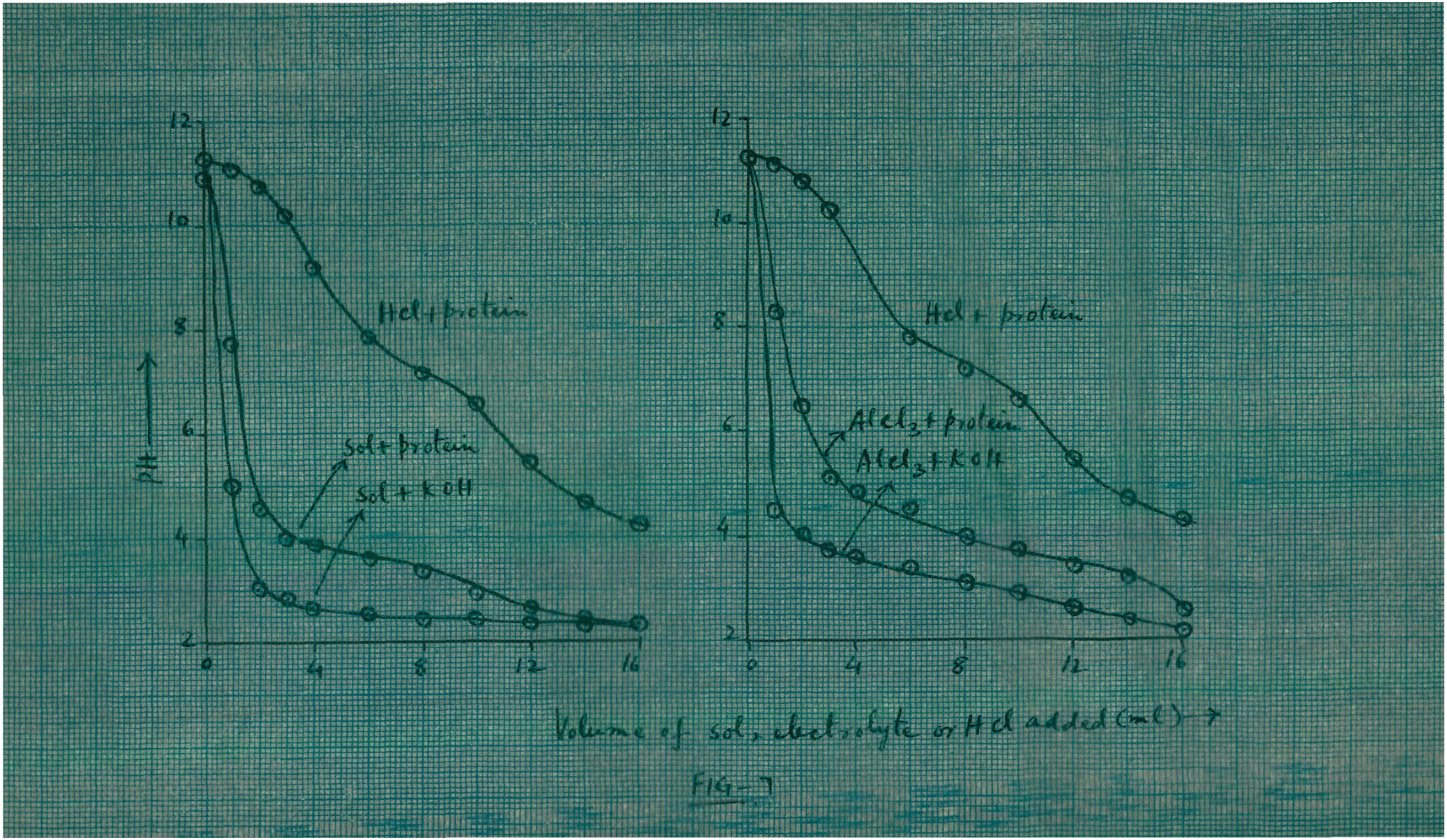
12
10
8
6
4
2
pH
0
4
8
12
16
HCl + protein
Sol + protein
Sol + KOH
HCl + protein
AlCl3 + protein
AlCl3 + KOH
Volume of sol, electrolyte or HCl added (in ml) →

FIG-1

TABLE 8.

Casein solution, concentration (2.8%), pH 12.0

Aluminium oxide sol, concentration (5.825 gm/L.) pH 2.0

Aluminium Chloride solution, concentration (2.12gm/L.),pH 2.0

Potassium hydroxide solution pH 12.0

Total volume 20 m.l. Temperature 30°C.

Volume of sol or electrolyte added	Aluminium oxide sol		Aluminium Chloride	
	pH with Casein	pH with KOH	pH with Casein	pH with KOH
0	10.95	11.00	10.95	11.00
1	7.75	5.00	8.25	4.50
2	4.55	3.00	6.41	4.00
3	4.00	2.85	5.00	3.70
4	3.90	2.65	4.80	3.60
6	3.55	2.55	4.50	3.40
8	3.35	2.45	3.95	3.05
10	2.90	2.40	3.70	2.90
12	2.62	2.35	3.38	2.56
14	2.45	2.30	3.18	2.35
16	2.30	2.25	2.55	2.15

(Fig. 7)

N.B. Metal ions were precipitated by alkali above pH 3.5, and sol coagulated in the visinity of pH 5 by casein.

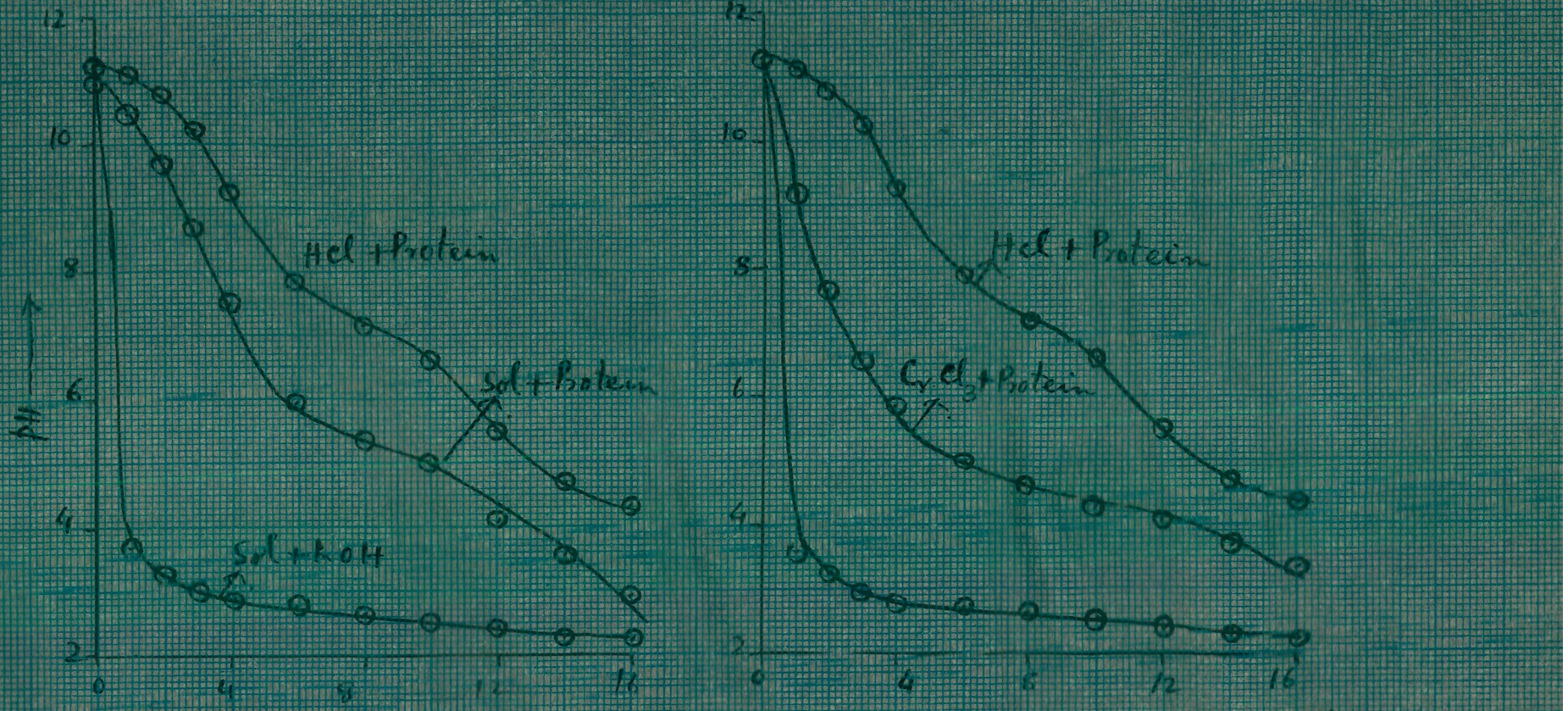
12
10
8
6
4
2
0
4
8
12
16
pH
HCl + Protein
Sol + Protein
Sol + KOH
12
16
8
4
0
4
8
12
16
HCl + Protein
CrCl3 + Protein
Volume of sol, electrolyte or HCl added (ml) →

FIG - 8.

TABLE 4.

Egg albumin solution, concentration (2.5%), pH 12.0

Potassium hydroxide solution pH 12.0

Hydro chloric acid solution pH 2.0

Total volume 20 m.l. Temperature 30°C

Volume of HCl added	pH with egg albumin	pH with KOH
0	11.10	11.50
1	10.75	8.50
2	10.45	4.05
3	9.70	3.90
4	9.05	3.70
6	7.32	3.55
8	6.50	3.20
10	5.00	3.05
12	4.25	2.60
14	3.85	2.40
16	3.55	2.20

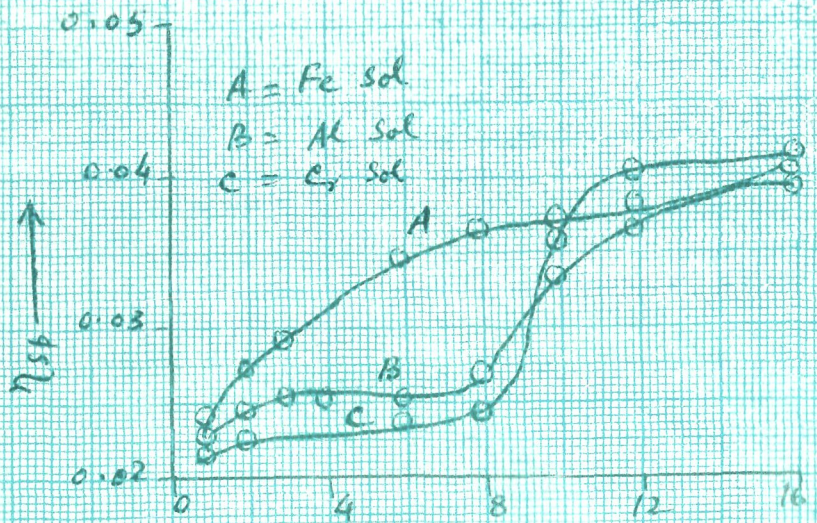

FIG-9

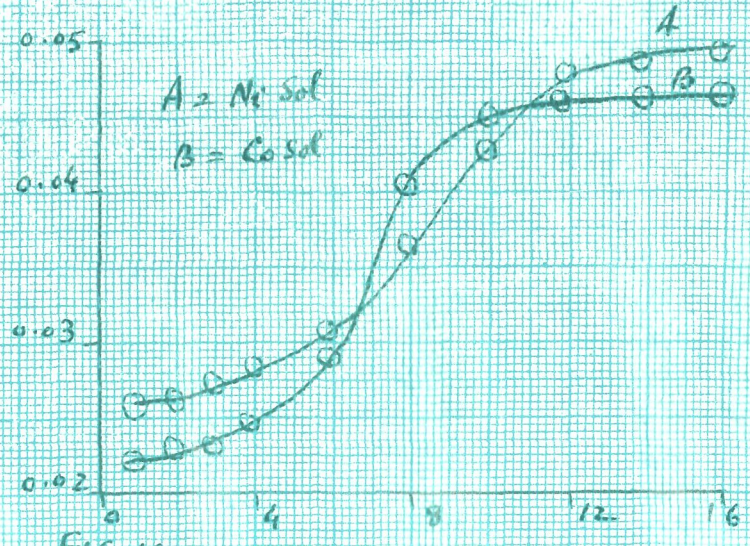

FIG-10

Volume of sol added (ml) →

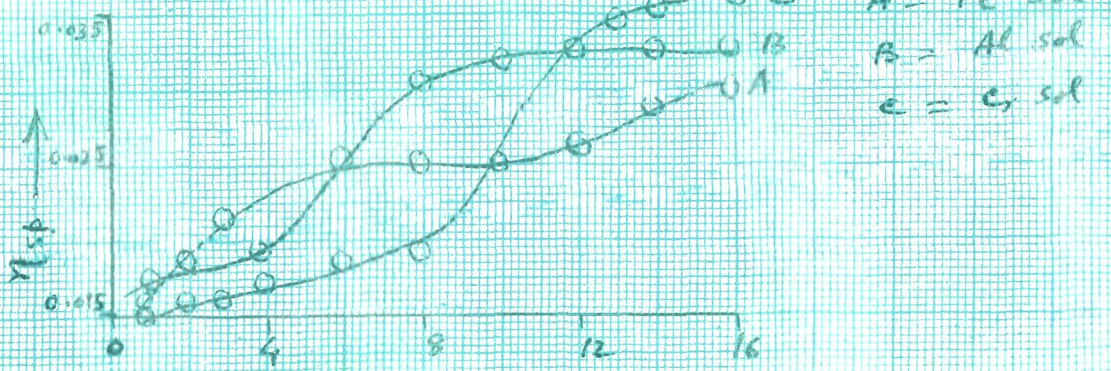

FIG-11. Volume of sol added (ml) →

TABLE 5.

Egg albumin solution, concentration (2.5%), pH 12.0

Nickel oxide sol, concentration (2.345 gm/L.), pH 7.6

Nickel Chloride solution, concentration (3.5×10^{-2}M) pH 7.6

Potassium hydroxide solution pH 12.0

Total volume 20 m.l. Temperature 30°C

Volume of sol or electrolyte added	Nickel oxide sol		Nickel Chloride	
	pH with egg albumin	pH with KOH	pH with egg albumin	pH with KOH
0	11.2	11.2	11.20	11.2
1	10.90	10.05	10.50	10.0
2	10.50	9.60	10.00	9.5
3	9.80	9.00	9.70	8.85
4	9.45	8.85	9.35	8.62
6	9.30	8.78	8.85	8.43
8	9.20	8.77	8.60	8.15
10	9.10	8.76	8.55	7.90
12	9.05	8.65	8.50	7.72
14	8.73	8.44	8.20	7.70
16	8.52	8.42	8.00	7.70

(Fig. 4)

N.B. The Alkali precipitated the metal ion and sol above pH 8.0, whereas no precipitation with anionic egg albamin.

TABLE 9.

Casein solution, concentration (2.8%), pH 12.0

Chromic oxide sol, concentration (0.945 gm/L.), pH 2.0

Chromic Chloride solution concentration (0.657gm/L.),pH 2.0

Potassium hydroxide solution pH 12.0

Total volume 20 m.l. Temperature 30°C.

Volume of sol or electrolyte added	Chromic oxide sol		Chromic Chloride	
	pH with Casein	pH with KOH	pH with Casein	pH with KOH
0	10.90	11.00	10.90	11.00
1	10.45	3.62	9.10	3.50
2	9.65	3.28	7.60	3.20
3	9.65	2.98	6.50	2.95
4	7.48	2.82	5.80	2.75
6	5.90	2.75	4.90	2.70
8	5.30	2.65	4.50	2.60
10	5.00	2.50	4.20	2.42
12	4.10	2.40	4.00	2.32
14	3.50	2.29	3.60	2.24
16	2.90	2.20	3.20	2.15

(Fig. 8)

N.B. Sol as well as metal ions were precipitated by the anionic protein at about 5 pH.

TABLE 10.

Casein solution, concentration (2.8%), pH 12.0

Potassium hydroxide solution pH 12.0

Hydrochloric acid solution pH 2.0

Total volume 20 m.l. Temperature 30°C

Volume of HCl added	pH with Casein	pH with KOH
0	11.2	11.50
1	11.05	8.50
2	10.75	4.05
3	10.20	3.90
4	9.20	3.70
6	7.85	3.55
8	7.15	3.20
10	6.55	3.05
12	5.45	2.60
14	4.65	2.40
16	4.25	2.20

TABLE 11.

VISCOSITY CHANGES

Egg albumin + ferric oxide sol system

Volume of sol added	n_s centipoise	n_m centipoise	$\frac{n_m - n_s}{n_s}$
1	0.8519	0.8723	0.240
2	0.8519	0.8752	0.0273
3	0.8519	0.8773	0.0290
6	0.8519	0.8814	0.0347
8	0.8519	0.8830	0.0365
10	0.8519	0.8835	0.0370
12	0.8519	0.8848	0.0384
14	0.8519	0.8852	0.0389
16	0.8519	0.8861	0.0400

(Fig. 9 A)

TABLE 12.

VISCOSITY CHANGES

Egg Albumin + Aluminium oxide sol system

Volume of sol added	n_s centipoise	n_m centipoise	$\frac{n_m - n_s}{n_s}$
1	0.8519	0.8713	0.0228
2	0.8519	0.8725	0.0242
3	0.8519	0.8735	0.0253
4	0.8519	0.8732	0.0250
6	0.8519	0.8732	0.0250
8	0.8519	0.8748	0.0267
10	0.8519	0.8802	0.0332
12	0.8519	0.8830	0.0365
16	0.8519	0.8853	0.0392

(Fig. 9 B)

T A B L E 13.

VISCOSITY CHANGES

Egg Albumin + Chromic oxide sol system

Volume of sol added	n_s centipoise	n_m centipoise	$\frac{n_m - n_s}{n_s}$
1	0.8519	0.8698	0.0209
2	0.8519	0.8713	0.0228
6	0.8519	0.8720	0.0235
8	0.8519	0.8725	0.0242
10	0.8519	0.8823	0.0357
12	0.8519	0.8865	0.0406
14	0.8519	0.8868	0.0408
16	0.8519	0.8870	0.0412

(Fig. 9 C)

T A B L E 14.

VISCOSITY CHANGES

Egg albumin+Nickel oxide sol system

Volume of sol added	n_s centipoise	n_m centipoise	$\frac{n_m - n_s}{n_s}$
1	0.8519	0.8738	0.0257
2	0.8519	0.8741	0.0260
3	0.8519	0.8749	0.0270
4	0.8519	0.8759	0.0281
6	0.8519	0.8778	0.0304
8	0.8519	0.8829	0.0363
10	0.8519	0.8880	0.0423
12	0.8519	0.8925	0.0476
14	0.8519	0.8930	0.0481
16	0.8519	0.8934	0.0487

(Fig. 10 A)

TABLE 15.

VISCOSITY CHANGES

Egg albumin + Cobalt oxide sol system

Volume of sol added	n_s centipoise	n_m centipoise	$\frac{n_m - n_s}{n_s}$
1	0.8519	0.8710	0.0220
2	0.8519	0.8715	0.0230
3	0.8519	0.8715	0.0230
4	0.8519	0.8726	0.0245
6	0.8519	0.8773	0.0290
8	0.8519	0.8861	0.0400
10	0.8519	0.8901	0.0448
12	0.8519	0.8908	0.0456
14	0.8519	0.8910	0.0458
16	0.8519	0.8910	0.0458

(Fig. 10 B)

TABLE 16.

VISCOSITY CHANGES

Casein + Ferric oxide sol system

Volume of sol added	n_s centipoise	n_m centipoise	$\frac{n_m - n_s}{n_s}$
1	0.8203	0.8335	0.0160
2	0.8203	0.8358	0.0188
3	0.8203	0.8382	0.0218
8	0.8203	0.8411	0.0252
10	0.8203	0.8412	0.0253
12	0.8203	0.8421	0.0265
14	0.8203	0.8438	0.0286
16	0.8203	0.8450	0.0300

(Fig. 11 A)

TABLE 17.

VISCOSITY CHANGES

Casein + Aluminium oxide sol system

Volume of sol added	n_s centipoise	n_m centipoise	$\frac{n_m - n_s}{n_s}$
1	0.8203	0.8347	0.0175
4	0.8203	0.8360	0.0191
6	0.8203	0.8413	0.0254
8	0.8203	0.8454	0.0306
10	0.8203	0.8465	0.0319
12	0.8203	0.8468	0.0323
14	0.8203	0.8473	0.0329
16	0.8203	0.8473	0.0329

(Fig. 11 B)

TABLE 18.

VISCOSITY CHANGES

Casein + Chromic oxide sol system

Volume of sol added	n_s centipoise	n_m centipoise	$\frac{n_m - n_s}{n_s}$
1	0.8203	0.8330	0.0154
2	0.8203	0.8335	0.0160
3	0.8203	0.8330	0.0164
4	0.8203	0.8347	0.0175
6	0.8203	0.8355	0.0185
8	0.8203	0.8360	0.0191
13	0.8203	0.8486	0.0345
14	0.8203	0.8492	0.0352
16	0.8203	0.8499	0.0365

(Fig. 11 C)

DISCUSSION

The interaction of positively charged sol with anionic proteins may be affected either by means of mutual adsopption of hydrophobic and hydrophilic colloidal particles or purely chemical forces may, however, be encountered, where the metal ions are made themselves available from the inner part of the double layer for combination with the reactive sites of protein molecule. Due account should be given, before any attempt is made to interpret the results, to the combination of hydrophobic sol through their counter ions (Chloride ions) if the protein under consideration, has the anion binding capacity.

pH-metric results

The pH-metric titrations carried out in presence of increasing amounts of iron, aluminium, chromium and their hydrous oxide sol with anionic casein give some conclusive evidence of binding of these ions with the available sites of protein. Since casein does not show any detectable chloride ion binding[44], the only explanation which may be offered for the variation in pH, is the possible chemical combination of metal ions with the protein.

It is evident from the figs(7 and 8) that the titration curves of casein in presence of hydrous oxide sols of chromium and aluminium lie above the corresponding curves of sol and alkali. Similar behaviour is observed for electrolyte-protein and electrolyte-alkali curves. Such results point towards the fact that protein can very well compete with its free base for metal ion binding. Moreover, the striking similarity between these curves is, that the flat portion lie between pH 3 - 5 indicating thereby that the ionised carboxyl groups[45] of casein play very significant role in the fixation of aluminium and chromium. Since the protein bears positive charge in this pH range, therefore the constancy of the pH can not be attributed to the mutual adsorption of the positively charged sol and the protein molecule. The amount with which the sol alkali or electrolyte alkali curves are displaced by the addition of protein to the sol or electrolyte may be taken as a qualitative measure of the extent of metal-protein combination. The order of combining power as indicated by Δ pH (figs 7 and 8), comes out to Cr $>$ Al. Similar explanation may be given to the behaviour of chromium, aluminium and their hydrous oxide sols towards egg albumin.

No definite information regarding the binding of iron to the egg albumin and casein is forthcoming by this method. A major portion of the ferric hydroxide sol-protein curve lie well below the corresponding

sol-alkali curve. In the vicinity of pH 10.5 the titration curves for ferric hydroxide sol with casein as well as with egg albumin give some qualitative test for metal ion binding, leading to the conclusion that iron may enter into combination with amino groups. Probably phenolic groups from tyrosine residue may also have participated in the interaction process, as has been shown by Schmidt[46] in case of casein and a number of related compounds.

The behaviour of cobalt and Nickel and their hydrous oxide sols towards egg albumin is quite interesting. However, the adsorptive effect can not be ruled out completely at such a high pH-values, though the nature of the titration curves (sol-protein and electrolyte protein curves lie above the sol-alkali and electrolyte-alkali curves), provide some useful informations regarding the binding of cobalt and nickel (from electrolyte as well as from sol) to the protein. The flat portion of these curves lie between pH 8 - 10 indicating thereby that the amino groups (probably imidazole groups also)may offer themselves as principal sites for metal ion binding.

Viscometric Results

The results obtained by viscometric measurements

are in accordance with those of pH-metric results. It is found that marked variation in viscosity takes place on adding gradually increasing amounts of sol to the proteins. The inflexion points occuring in the viscosity concentration curves are rather striking.. These points occur at those pH-values where chemical combination is indicated on the basis of pH-metric measurements. Assuming that the abrupt change in specific viscosity represents a new state of aggregation, as pointed out by P. Doty[32], these inflexion points in specific viscosity-concentration curves (Figs 9, 10 and 11) lead one to the following conclusions

(i) Chromium and aluminium both appear to bind with the carboxyl groups of egg albumin as well as with casein. The binding capacity (as calculated on the basis of Δ pH (Figs 3 and 8) of individual protein for Cr and aluminium increases in the order,

Egg albumin $<$ Casein.

it seems plausible in view of the amino acid composition of the two proteins[45,47] where as chromium has got greater binding capacity as compared to aluminium for a given protein.

(ii) No definite evidence regarding the binding of ferric ions to the carboxyl groups of both the protein is forthcoming. On the other hand the flat portion of the titration curve of both the protein slightly

indicate the possibility of combination of iron to either the E-amino groups from lysyl residue or to the phenolic groups of tyrosine residue, since these flat portion of the curves exhibit the constancy in pH in the region where E-amino groups,(possibly phenolic groups also may become reactive due to deprotonation.

(iii) Cobalt and nickel appear to combine with the amino groups (imidazole groups may also involve) of egg albumin. Although the possibility of adsorption may not be ruled out completely.

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P A R T IV.

Potentiometric studies on the interaction of Lead and Chromium with amino acids and related compound.

I N T R O D U C T I O N

It is generally accepted, that the role of metal ions in biochemical processes is a mere reflection of their tendency to undergo complex ion formation[1] with the active sites of proteins or to the various functional groups of enzyme. The specificity of the metal ions is directly linked with the stability of these complexes, and the catalytic activity usually associated with the metal ions in enzyme reaction manifests itself through an intermediate stage, involving metal complexes. The work of Hellerman and his associates[2] had provided remarkable support to this view point, and several metals of the transition series are known to catalyse the decarboxylation of dimethyloxalacetic acid through metal-chelate formation.[3] Pedersen[4], on the basis of kinetic studies of the rate of clevage of nitroacetic acid in presence of metal ions, has demonstrated that the decrease in the rate of reaction in presence of copper is due to the formation of metal nitroacetate complex. The inhibition of glucose metabolism in yeast cell by UO_2^{++} ions[5], the activation of enzymes[6] extracted from Aspergillusniger by Zn^{++} and Mn^{++} and the inhibition of alkaline phosphatase[7] by Be^{++} ions are good examples of biochemical systems involving complex formation.

The existing literature on this particular field, although supplies valuable information about complexes of the type given above; yet some aspects of the problem dealing with the affinity of different naturally occuring complexing agents for metal ions of biological importance have to be worked out. With this aim in view, the present investigations were carried out on the composition and stability of metal complexes, obtained by interaction with simple building units of proteins i.e. amino acids and peptide employing Bjerrum's method.[8] The metal chosen for these studies were Pb(ii) and Cr(iii).

Bjerrum's work, "Metal Ammine Formation in Aqueous Solution", introduced a new epoch in the Chemistry of metal complexes. He was the first to emphasise that complex formation follows an stepwise course. Considering the equilibrium between a metal ion 'M' and Ligand 'A' the following steps may be visualised

$$M + A \rightleftharpoons MA$$

$$MA + A \rightleftharpoons MA_2$$

$$\cdots\cdots\cdots\cdots$$

$$MA_{N-1} + A \rightleftharpoons MA_N$$

(step equilibria like above are found in case of acid base equilibrium; in redox system[9,10] and in case of complex formation). The individual formation constants may be given according to the law of mass action

$$K_1 = \frac{(MA)}{(M)(A)}$$

$$K_2 = \frac{(MA_2)}{(MA)(A)}$$

$$K_N = \frac{(MA_N)}{(MA_{N-1})(A)}$$

where the values given in parentheses are the concentration terms. According to Bjerrum the concentration constants of a system may be calculated when $\bar{n}$, average number of ligand molecule attached to 'M' as a function of the concentration of free ligand, is known. $\bar{n}$ may be given as

$$\bar{n} = \frac{(MA) + 2(MA_2) + \ldots\ldots N(MA_N)}{(M) + (MA) + (MA_2) + \ldots\ldots (MA_N)}$$

The value of $\bar{n}$ is determined experimentally by measuring the pH. Since the removal of free donor groups by coordination alters the pH by the release of corresponding number of protons, therefore, the amount with which the pH is shifted in a mixture of known quantities of ligand and metal ions, may be used to calculate the number of groups coordinated. The difference in the concentration of added, and coordinated ligands gives the concentration of free ligand (A). $\bar{n}$ is also known as formation function; hence a plot of $\bar{n}$ against - log (A) is termed as formation curve.

Bjerrum's method has been extensively used by a number of workers in the field of coordination chemistry. Flood and Loras[11] have investigated glycinate complexes with a number of bivalent metal ions (Viz., Co. Ni, Zn, Cu, Hg and Ag.). Maley and Mellor[12] have studied the copper zinc, cobalt and manganese complexes of glycine, alanine,

valine, histidine and Leucine. Albert made a systematic study of a number of metal ions with amino acids having two[13] and three[14] ionising groups. De-Ghosh and Ray[15] have employed Bjerrum's method for the calculation of stability constants of cobalt biguanide complexes. C.B. Monk[16] has also utilised this method to study the complexes of glycine, alanine and glycylglycine, with a number of metal ions (Cu, Ag, Pb, Ni, Zn, Co, Mn, Mg and Ca). Electrostatic effect on the combination of cobalt ions with glycine, alanine, asparagine and arginine were investigated by Tanford and Shore[17]. Perkins[18] used Bjerrum's method as illustrated by the work of Albert[13] to study the effect of amino acid structure on the stability constants of the complexes formed with metals of groups II of the periodic table. N.C.Li and coworkers[19-20] have investigated systematically, the metal complexes of amino acids with sulphur containing groups. Robin, Leberman and Data[22-24] have extended the conventional pH metric method to the metal-peptide complexes. Green and Ang[25] made an indirect approach towards the chromium alanine complex. More recently Malik and Khan[26] studied the chromium complexes of amino acids having two ionising groups.

EXPERIMENTAL

Apparatus and Technique

pH measurements were carried out by means of Backman Model G pH-metric using glass electrod; pH-metric was standardised against .05M potassium hydrogen phthalate, 0.05M sodium borate and standard Backman buffer (pH 7.0). The titration cell was kept in a water thermostate (Townson and Mercur Corydon Ltd.) to maintain constant temperature and nitrogen (purified by passing through alkaline pyrogallol and chromous chloride) was passed through the reaction mixture to ensure an innert atmosphere.

Reagents and Solutions

Carborate free potassium hydroxide was prepared as described earlier, its concentration determined by pH-metric titration against standard potassium hydrogen phthalate. A.R. sample of hydrochloric acid was used to prepare the dilute solution, its concentration determined by the usual method.

Lead nitrate (E.Merck) and chromic chloride (Baker A.R.) were used as source of metal ions, their solution were prepared in doubly distilled water and concentration determined as described earlier. A.R. sample of potassium chloride and potassium nitrate were dissolved in doubly distilled water to get their respective solutions. These

solutions were used to maintain the constant ionic strength.

Solutions of amino acids were prepared by dissolving an accurately weighed sample of amino acid in doubly distilled water.

Procedure:

Lead nitrate (0.5×10^{-3}M), with excess amount of various amino acids (4.0×10^{-3}M, Glycine, proline, Asparagine Lysine, Valine, Threonine, Phenylalanine, Serine, Methionine and Histidine and 5.0×10^{-3}M, Hydroxy proline) was titrated by carbonate free potassium hydroxide. The total volume was kept to 50 m.l., and sufficient quantity of potassium nitrate was added to maintain the constant ionic strength (0.15) in each case. Small amounts of alkali were added at a time, and pH was recorded after each addition. All measurements were carried out at 25 $\pm$ 0.1°C.

Chromic chloride (0.4×10^{-3}M), with excess amount of various amino acids (4.0×10^{-3}M, glycine, proline, threonine, Phenylalanine, Histidine and glycylglycine, and 5.0×10^{-3}M Hydroxy proline) was refluxed for about two and half hours. A light pink colour was developed in each cases, then the mixtures were cooled and diluted to 50 m.l. with the addition of water and requisite amount of potassium chloride so as to maintain the constant ionic strength (0.15). All the mixtures were titrated afterward as lead amino acid complexes, under identical conditions. The results are summarised in the following tables.

TABLE 1.

pH-metric titration of L-proline and lead nitrate

Base($\bar{O}H$) added $x10^{-3}$ M	pH	log (A)	$\bar{n}$	log K'	log K''
0	6.00	- -	-	-	-
0.1996	6.32	$\bar{7}$.2198	0.100	5.82	-
0.3984	6.80	$\bar{7}$.2764	0.200	6.12	-
0.5964	7.10	$\bar{7}$.9519	0.298	5.65	-
0.9902	7.48	$\bar{6}$.2584	0.495	5.81	-
1.5750	7.85	$\bar{6}$.5549	0.787	5.99	-
1.9610	8.32	$\bar{6}$.9494	0.980	-	-
2.3440	8.60	$\bar{5}$.1338	1.170	-	4.17
2.7240	9.00	$\bar{5}$.4259	1.360	-	4.31
2.2880	9.80	$\bar{5}$.9725	1.640	-	4.27

TABLE 2.

pH-metric titration of Glycine and lead nitrate

Base ($\bar{O}H$) added $x10^{-3}$ M	pH	log (A)	$\bar{n}$	log K'	log K''
0	5.90	-	-	-	-
0.1996	6.28	$\bar{7}$.9998	0.10	5.04	-
0.3984	6.72	$\bar{6}$.4165	0.20	4.98	-
0.5964	6.92	$\bar{6}$.5919	0.29	5.04	-
0.9902	7.15	$\bar{6}$.7684	0.49	5.23	-
1.5750	7.77	$\bar{5}$.2941	0.78	5.15	-
1.9610	7.92	$\bar{5}$.3694	0.98	-	-
2.3440	8.20	$\bar{5}$.5591	1.17	-	3.75
2.7240	8.50	$\bar{5}$.7459	1.36	-	4.00
2.880	8.76	$\bar{5}$.9425	1.64	-	4.30

TABLE 3.

pH-metric titration of Hydroxy proline and lead nitrate

Base($O\bar{H}$) added $\times 10^{-3}$ M	pH	log(A)	$\bar{n}$	log K′	log K″
0	5.62	-	-	-	-
0.3984	6.23	$\bar{6}$.1629	0.16	5.11	-
0.5964	6.55	$\bar{6}$.4638	0.24	5.04	-
0.9902	6.80	$\bar{6}$.6731	0.40	5.15	-
1.5750	7.35	$\bar{5}$.1546	0.63	5.08	-
1.9610	7.52	$\bar{5}$.2727	0.78	5.26	-
2.3440	7.63	$\bar{5}$.3242	0.94	-	-
2.7240	8.00	$\bar{5}$.5272	1.10	-	3.52
3.2880	8.35	$\bar{5}$.8535	1.30	-	3.78

TABLE 4.

pH-metric titration of L-Asparagine and lead nitrate

Base($O\bar{H}$) added $\times 10^{-3}$ M	pH	log(A)	$\bar{n}$	log K′	log K″
0	5.35	-	-	-	-
0.1996	5.50	$\bar{6}$.2298	0.10	4.82	-
0.3984	5.85	$\bar{6}$.5564	0.20	4.84	-
0.5964	6.20	$\bar{6}$.8819	0.29	4.72	-
0.9902	6.52	$\bar{5}$.1484	0.49	4.83	-
1.5750	7.16	$\bar{5}$.6847	0.78	4.85	-
1.9610	7.40	$\bar{5}$.8594	0.98	-	-
2.3440	7.65	$\bar{4}$.0191	1.17	-	3.29
2.7240	7.85	$\bar{4}$.1059	1.36	-	3.64
3.2880	8.70	$\bar{4}$.7025	1.64	-	3.54

TABLE 5.

pH-metric titration of DL. Lysine and lead nitrate

Base $(O\bar{H})$ added x 10^{-3} M	pH	log(A)	$\bar{n}$	log K′	log K″
0	5.25	-	-	-	-
0.1996	5.65	$\bar{6}$.2798	0.10	4.76	-
0.3984	5.98	$\bar{6}$.5864	0.20	4.51	-
0.5964	6.35	$\bar{6}$.9319	0.29	4.67	-
0.9902	6.68	$\bar{5}$.2084	0.49	4.77	-
1.5750	7.32	$\bar{5}$.7547	0.78	4.90	-
1.9610	7.55	$\bar{5}$.9094	0.98	-	-
2.3440	7.85	$\bar{4}$.1191	1.17	-	3.19
2.7240	8.15	$\bar{4}$.3059	1.36	-	3.40
3.2880	8.90	$\bar{4}$.8025	1.64	-	3.44

TABLE 6.

pH-metric titration of DL. Valine and lead nitrate

Base $(O\bar{H})$ added x 10^{-3} M	pH	log(A)	$\bar{n}$	log K′	log K⁴
0	6.15	-	-	-	-
0.1996	6.40	$\bar{6}$.2598	0.10	4.78	-
0.3984	6.90	$\bar{6}$.7364	0.20	4.66	-
0.5964	7.15	$\bar{6}$.9619	0.29	4.64	-
0.9902	7.50	$\bar{5}$.2584	0.49	4.72	-
1.5750	8.10	$\bar{5}$.7647	0.78	4.78	-
1.9610	8.25	$\bar{5}$.8694	0.98	-	-
2.3440	8.50	$\bar{5}$.9991	1.17	-	3.31
2.7240	8.90	$\bar{4}$.4859	1.36	-	3.26
3.2880	9.68	$\bar{4}$.8125	1.64	-	3.43

TABLE 7.

pH-metric titration of threonine and lead nitrate

Base $(\bar{O}H)_{3}$ added $x10^{-3}$M	pH	log(A)	$\bar{n}$	log K′	log K″
0	6.1	-	-	-	-
0.1996	6.48	$\bar{6}$.7498	0.10	4.29	-
0.3984	6.87	$\bar{5}$.0764	0.20	4.32	-
0.5962	7.20	$\bar{5}$.3819	0.29	4.22	-
0.9902	7.53	$\bar{5}$.7584	0.49	4.22	-
1.5750	8.10	$\bar{4}$.1347	0.78	4.41	-
1.9610	8.28	$\bar{4}$.2394	0.98	-	-
2.3440	8.50	$\bar{4}$.3691	1.17	-	2.94
2.7240	8.82	$\bar{4}$.5759	1.36	-	3.17
3.2880	9.49	$\bar{4}$.9925	1.64	-	3.25

TABLE 8.

pH-metric titration of DL. Phenylalanine and lead nitrate

Base $(\bar{O}H)_{3}$ added $x10^{-3}$M	pH	log(A)	$\bar{n}$	log K′	log K″
0	6.20	-	-	-	-
0.1996	6.50	$\bar{6}$.7698	0.10	4.27	-
0.3984	6.90	$\bar{5}$.1464	0.20	4.25	-
0.5964	7.22	$\bar{5}$.4419	0.29	4.17	-
0.9902	7.53	$\bar{5}$.6984	0.49	4.28	-
1.5750	8.15	$\bar{4}$.2247	0.78	4.32	-
1.9610	8.30	$\bar{4}$.2990	0.98	-	-
2.3440	8.50	$\bar{4}$.4091	1.17	-	2.90
2.7240	8.85	$\bar{4}$.6459	1.36	-	3.10
3.2880	9.50	$\bar{3}$.0425	1.64	-	3.20

TABLE 9.

pH-metric titration of DL. Serine and lead nitrate

Base $(O\bar{H})_{-3}$ M added x10^{-3}	pH	log(A)	$\bar{n}$	log K′	log K″
0	6.0	-	-	-	-
0.1996	6.45	$\bar{6}$.7898	0.10	4.25	-
0.3984	6.87	$\bar{5}$.1864	0.20	4.21	-
0.5964	7.20	$\bar{5}$.4919	0.29	4.11	-
0.9902	7.55	$\bar{5}$.7884	0.49	4.19	-
1.5750	8.18	$\bar{4}$.3247	0.78	4.22	-
1.9610	8.30	$\bar{4}$.3694	0.98	-	-
2.3440	8.45	$\bar{4}$.4291	1.17	-	2.88
2.7240	8.87	$\bar{4}$.7359	1.36	-	3.01
3.2880	9.54	$\bar{3}$.1525	1.64	-	3.09

TABLE 10.

pH-metric titration of DL. Methionine and lead nitrate

Base $(O\bar{H})_{-3}$ M added x10^{-3}	pH	log(A)	$\bar{n}$	log K′	log K″
0	6.24	-	-	-	-
0.1996	6.65	$\bar{6}$.8898	0.10	4.15	-
0.3984	7.04	$\bar{5}$.2564	0.20	6.14	-
0.5964	7.36	$\bar{5}$.5519	0.29	4.05	-
0.9902	7.68	$\bar{5}$.8184	0.49	4.16	-
1.5750	8.30	$\bar{4}$.3447	0.78	4.20	-
1.9610	8.46	$\bar{4}$.4294	0.98	-	-
2.3440	8.62	$\bar{4}$.4991	1.17	-	2.88
2.7240	9.05	$\bar{4}$.8159	1.36	-	2.90
3.2880	9.60	$\bar{3}$.1125	1.64	-	3.10

TABLE 11.

pH-metric titration of DL. Histidine and lead nitrate

Base ($\bar{O}H$) added $x10^{-3}$ M	pH	log(A)	$\bar{n}$	log K′	log K″
0	3.30	-	-	-	-
0.1996	3.50	$\bar{6}.9798$	0.10	4.06	-
0.3984	3.75	$\bar{5}.2064$	0.20	4.19	-
0.5964	4.10	$\bar{5}.5319$	0.29	4.07	-
0.9902	4.45	$\bar{5}.8284$	0.49	4.15	-
1.5750	5.10	$\bar{4}.3847$	0.78	4.16	-
1.9610	5.25	$\bar{4}.4594$	0.98	-	-
2.3440	5.40	$\bar{4}.5119$	1.17	-	2.79
2.7240	6.02	$\bar{3}.0259$	1.30	-	2.72
3.2880	6.58	$\bar{3}.4025$	1.64	-	2.78

(Fig.)

TABLE 12.

pH-metric titration of L.proline and Cr(iii)

Base ($\bar{O}H$) added $x10^{-3}$ M	pH	log(A)	$\bar{n}$	log K′	log K″
0	3.30	-	-	-	-
0.3984	3.60	$\bar{10}.4764$	0.20	8.92	-
0.5962	3.90	$\bar{10}.7519$	0.29	8.86	-
0.9902	4.15	$\bar{10}.9484$	0.49	9.04	-
1.5750	4.80	$\bar{9}.5057$	0.78	9.04	-
1.9610	5.20	$\bar{9}.8294$	0.98	-	-
2.3440	5.60	$\bar{8}.1391$	1.17	-	7.17
2.7240	5.98	$\bar{8}.4059$	1.36	-	7.34
3.2880	6.70	$\bar{8}.8725$	1.64	-	7.37
3.8460	7.50	$\bar{7}.0075$	1.92	-	-

(Fig.)

TABLE 13.

pH-metric titration of Glycine and Cr(iii)

Base($\bar{O}H$)	pH	log(A)	$\bar{n}$	log K′	log K″
0	3.41	-	-	-	-
0.3984	3.80	$\bar{9}$.4964	0.20	7.90	-
0.5962	4.18	$\bar{9}$.8519	0.29	7.75	-
0.9902	4.48	$\bar{8}$.0984	0.49	7.88	-
1.5750	5.00	$\bar{8}$.5247	0.78	8.02	-
1.9610	5.70	$\bar{7}$.1494	0.98	-	-
2.3440	5.90	$\bar{7}$.2591	1.17	-	6.05
2.7240	6.30	$\bar{7}$.5459	1.36	-	6.20
3.2880	7.00	$\bar{7}$.9925	1.64	-	6.25

TABLE 14.

pH-metric titration of Hydroxy proline and Cr(iii)

Base ($\bar{O}H$) added $x10^{-3}$ M	pH	log(A)	$\bar{n}$	log K′	log K″
0	3.0	-	-	-	-
0.3984	3.35	$\bar{9}$.2829	0.16	7.99	-
0.5962	3.60	$\bar{9}$.5138	0.24	7.98	-
0.9902	3.90	$\bar{9}$.7731	0.20	8.05	-
1.5750	4.50	$\bar{8}$.3045	0.63	7.92	-
1.9610	4.90	$\bar{8}$.6527	0.78	7.89	-
2.3440	5.40	$\bar{7}$.0942	0.94	-	-
2.7240	5.70	$\bar{7}$.3272	1.10	-	5.72
3.2880	6.10	$\bar{7}$.6035	1.30	-	6.03
3.8460	6.80	$\bar{6}$.1322	1.54	-	5.93

TABLE 15.

pH-metric titration of Threonine and Cr(iii)

Base $(O\bar{H})$ added $x10^{-3}$ M	pH	log(A)	$\bar{n}$	log K′	log K″
0	3.12	-	-	-	-
0.3984	3.61	$\bar{9}$.8164	0.20	7.58	-
0.5962	3.93	$\bar{8}$.1119	0.29	7.49	-
0.9902	4.50	$\bar{8}$.6284	0.49	7.35	-
1.7550	5.00	$\bar{7}$.0347	0.78	7.51	-
1.9610	5.44	$\bar{7}$.3994	0.98	-	-
2.3440	6.21	$\bar{6}$.0791	1.17	-	5.23
2.7240	6.80	$\bar{6}$.5559	1.36	-	5.19
3.2880	7.48	$\bar{6}$.9825	1.64	-	5.26

TABLE 16.

pH-metric titration of DL. Phenylalanine and Cr(iii)

Base $(O\bar{H})$ added $x10^{-3}$ M	pH	log(A)	$\bar{n}$	log K′	log K″
0.	3.05	-	-	-	-
0.3984	3.60	$\bar{9}$.8464	0.20	7.55	-
0.5962	3.92	$\bar{8}$.1419	0.29	7.46	-
0.9902	4.48	$\bar{8}$.6486	0.49	7.33	-
1.5750	5.00	$\bar{7}$.0747	0.78	7.47	-
1.9610	5.45	$\bar{7}$.4491	0.98	-	-
2.3440	6.20	$\bar{6}$.1091	1.17	-	5.00
2.7240	6.80	$\bar{6}$.5959	1.36	-	5.15
3.2880	7.50	$\bar{5}$.0425	1.64	-	5.20

TABLE 17.

pH-metric titration of Glycyl-glycine and Cr(iii)

Base $(O\bar{H})_{-3}$ added x10^{-3}M	pH	log(A)	$\bar{n}$	log K′	log K″
0	3.50	-	-	-	-
0.3984	3.81	$\bar{7}$.2963	0.20	6.10	-
0.5962	4.25	$\bar{7}$.5119	0.29	6.09	-
0.9902	4.60	$\bar{7}$.8084	0.49	6.18	-
1.5750	5.15	$\bar{6}$.2647	0.78	6.29	-
1.9610	5.82	$\bar{6}$.8594	0.98	-	-
2.3440	6.05	$\bar{5}$.9991	1.17	-	4.31
2.7240	6.53	$\bar{5}$.3659	1.36	-	4.38
3.2880	6.16	$\bar{5}$.8425	1.64	-	4.40

TABLE 18.

pH-metric titration of DL.Histidine and Cr(iii)

Base $(O\bar{H})_{-3}$ added x10^{-3}M	pH	log(A)	$\bar{n}$	log K′	log K″
0	3.0	-	-	-	-
0.3984	3.28	$\bar{6}$.7364	0.20	4.66	-
0.5962	3.60	$\bar{5}$.2119	0.29	4.58	-
0.9902	3.90	$\bar{5}$.2784	0.49	4.70	-
1.5750	4.55	$\bar{5}$.8347	0.78	4.71	-
1.9610	4.70	$\bar{5}$.9094	0.98	-	-
2.3440	4.90	$\bar{4}$.0191	1.17	-	3.29
2.7240	5.50	$\bar{4}$.5059	1.36	-	3.24
3.2880	6.20	$\bar{4}$.9525	1.64	-	3.30

RESULTS AND DISCUSSION

The pH-values obtained by the addition of alkali to the mixtures of lead nitrate or chromic chloride and various amino acids have been recorded in the preceding tables (Tables 1 - 18 column 2). From these readings, the values for $\bar{n}$, the average number of molecules of complex forming agent bound by one atom of metal and (A), the concentration of free chelating agent (in the anionic form) were determined following Albert method[13],(A) may be given as

$$\log(A) = (pH - pka) + \log\left((HA) - (KOH)\right) \quad \ldots (1)$$

where (HA) is the concentration of complex-forming agent (all species), (KOH) is the concentration of potassium hydroxide and pka is the second ionisation constant of amino acid. The pka values of Glycine, L-proline, L-Asparagine. DL-valine, DL-Phenylalanine, DL-Serine and DL-Mathionine were taken from those obtained by Albert[13], and those of L-lysine, L-Histidine, L-Hydroxy proline and glycylglycine were taken from Schmidt[27], Greenstein[28], Kirk[29] and Datta[23] respectively. Threonine was worked out under the experimental condition and pka was found to be 9.35. The values of log K' and log K'' given in column 5 and 6 (Tables 1 to 18) refer to the stepwise stability constants, these were calculated from the values of log(A) at $\bar{n}$ = 0.1 to 0.8 and $\bar{n}$ = 1.2 to 1.7 respectively employing the following expressions[13]

$$K' = \frac{\bar{n}}{(1-\bar{n})(A)} \quad \ldots\ldots\ldots (2)$$

$$K'' = \frac{(\bar{n} - 1)}{(2 - \bar{n})(A)} \quad \ldots\ldots\ldots (3)$$

Formation curves were obtained by plotting $\bar{n}$-against log(A). From such curves it was found that all the α-amino acid reported here gave stepwise addition with lead as well as with chromium. The overall stability constants K_s, for all the complexes were calculated using the relationship.

$$\log K_s = -2 \log (A) \quad \ldots\ldots\ldots (4)$$

where log(A) is the concentration of free complex forming agent (in the anionic form) corresponding to $\bar{n} = 1$. Since log(A) at $\bar{n} = 1$ does not necessarily available from direct pH-titration, therefore, these values were obtained from the formation curves. Log K_s-values thus calculated are listed in tables 19 and 20 (Column 4). The values given in parentheses in the same column are those obtained by combining log K' and log K'' according to the equation (Albert[13])

$$K_s = K'.K'' \quad \ldots\ldots\ldots (5)$$

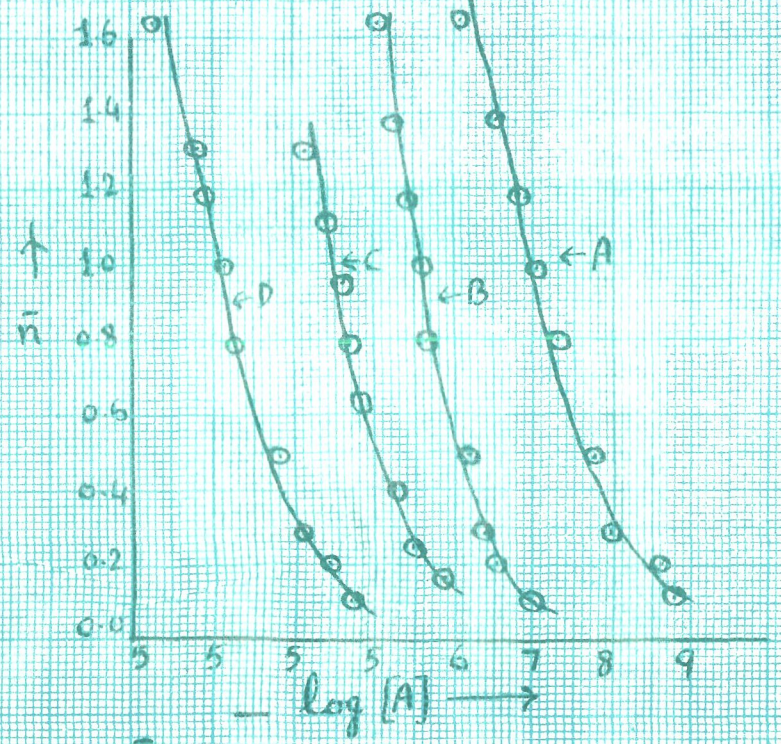

Fig 1.
Formation Curves. Each Curve starts at 5
A. Lead - proline; B. Lead glycine
C. Lead - hydroxy proline.
D. Lead - asparagine.

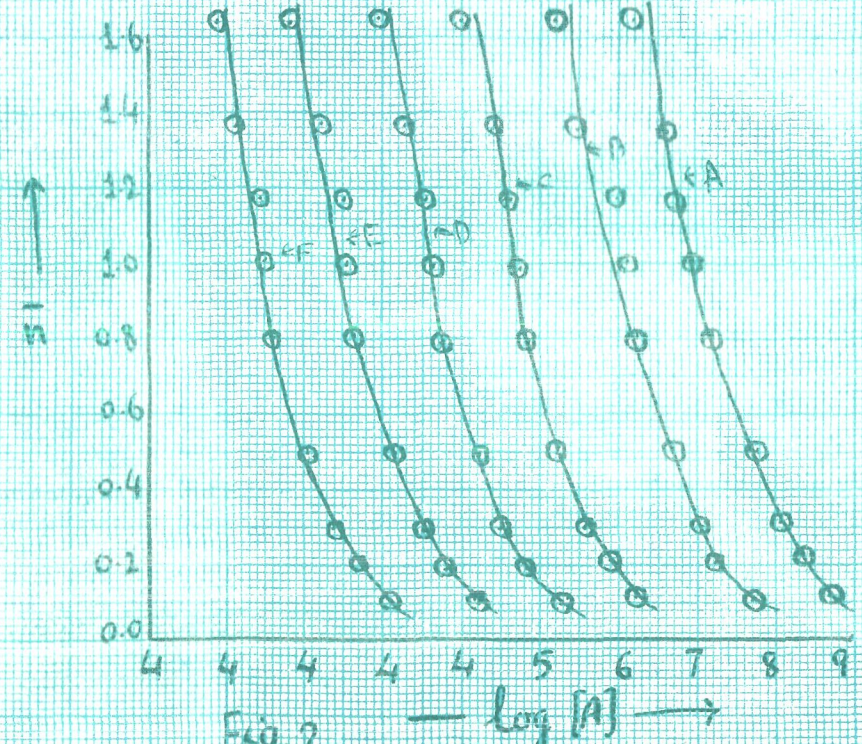

Fig 2.
Formation Curves. Each Curve starts at 4.
A. Lead - lysine; B. Lead Valine
C. Lead - threonine D. Lead Phenyl alanine
F. Lead - methionine.

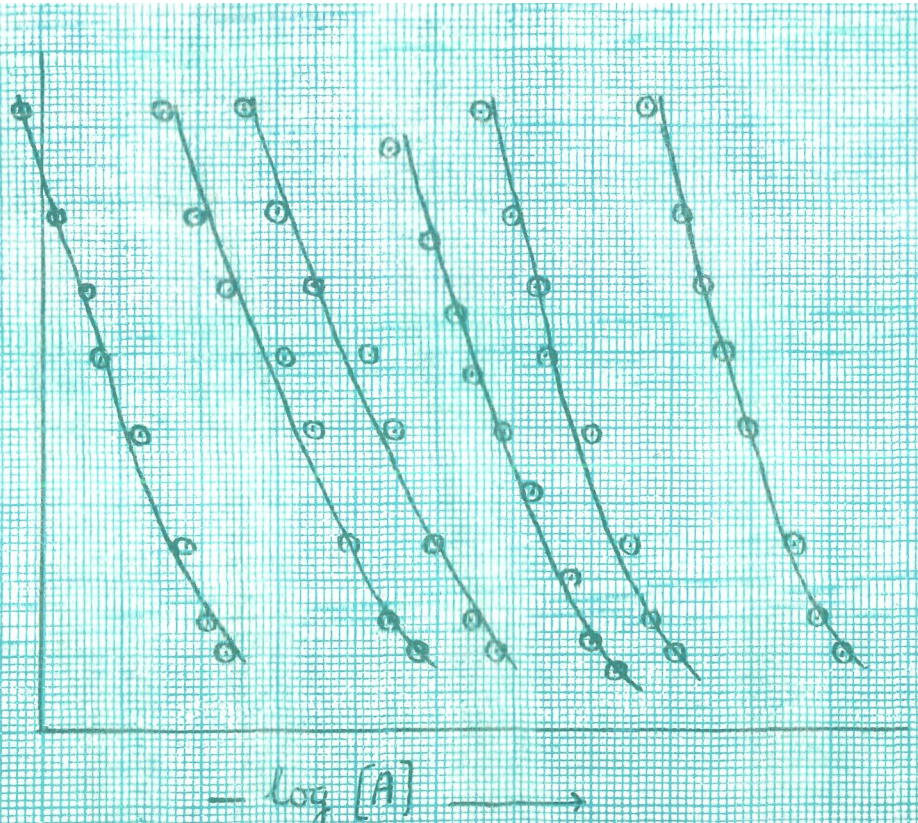

Fig 3

Formation Curves ... Each Curve-start at 6
A. Cr-proline; B. Cr-glycine; C. Cr-hydroxy proline
D. Cr-Threonine; E. Cr-phenyl alanine
F. Cr-glycyl glycine.

Lead and chromium both when titrated against alkali in presence of various amino acids, remain suspended beyond the pH at which normally metal hydroxides precipatate out.[30] Lead can be titrated upto pH 8.5 where as chromium upto 7.5, When the pH exceeds this value, the precipitation of hydroxides sets in. Moreover, the pink colour of most of the chromic complexes changes to green before precipitation. These preliminary observations lead to the conclusion that the formation of metal hydroxide has been prevented due to the strong binding of metal ions to the chelating agents. Furthermore the chromium complexes seem to undergo gradual decomposition during the process of titration.

The formation curves obtained by plotting - log(A) against $\bar{n}$ are valuable because they provide necessary information regarding, that whether the reaction under consideration is stepwise or not. From these curves, it is evident that all the amino acids reported here (Tables 19 & 20), form stepwise complexes with lead as well as with chromium The values of log(A) at $\bar{n}$ = 1 were obtained from these curves and overall stability constants were calculated. From these values it is possible to draw a number of generalisations.

(i) Log K_s-values calculated from the equation log K_s = -2 log(A) (where log(A) is the value at $\bar{n}$ = 1) agree well with those obtained by combining stepwise stability constants (These values are given in parentheses column 4, tables

19 and 20). This agreement in log K_s values further supports the view, that amino acids form the stepwise complexes.[13]

(ii) It has been found that the values of stability constants of various lead amino acid complexes lie between the values of corresponding Zinc and Cobalt complexes[13]. This confirms the order Ni > Zn > Pb > Co for the complexing tendency of bivalent metal ions to a series of - amino acids, reported by Monk and Albert.[13] It is also interesting to note that the above mentioned metal ions follow a different order i.e. Ni > Pb > Co > Zn for salicyl aldehyde, ethyldiamine and other organic ligands[12]. In fact some irregularities are apparent when the log K_s values reported in the literature[13] for Cobalt amino acids complexes are compared with those for lead-amino acids complexes obtained during the present investigations. The order given by Monk seems to be even reversed for amino acid containing carbonyl (Asparagine) hydroxyl (Serine) and thio ether (Methionine) groupings. The typical values are shown here.

	Log K_s		
	Zn	Pb	Cp
Glycine	9.3	9.2	8.9
L-Proline	10.2	10.1	9.3
Serine	-	7.26	8.0
Asparagine	9.7	8.28	8.4
Methionine	-	7.14	7.9

(The values for Zn and Co are taken from Albert)[13]

(iii) The difference in over all stability constants for various lead and chromium amino acid complexes is not strikingly large. This leads to the conclusion that lead-amino acid linkage in various α-amino acids may not be radically different, and also chromium may form identical linkage with various α-amino acids.[26] However, proline appears to have greatest affinity for both the metal ions, and the rest of the amino acids follow the order for Pb(ii) (log K_s values are given in parentheses).

Proline (10.1) > Glycine (9.2) > Hydroxy proline (9.1) > Asparagine (8.28) > Lysine (8.18) > Valine (8.2) Threonine (7.52) > Phenyl alanine (7.4) > Serine (7.26) > Metheonine (7.14)

and for Cr(iii)

Proline (16.3) > Glycine (13.7) > Hydroxy proline (13.6) > Threonine (13.1) > Phenyl alanine (13.0) > Glycylglycine (10.3).

This order revealsthe following information regarding the influence of ligand structure upon chelation; (i) the increase in the length of carbon chain greatly decreases the stability constant, (ii) the introduction of a bulky $-CH_2-\langle\!=\!\rangle$ group at the α-carbon atom brings about some negative influence on complex formation, and (iii) the introduction of OH group in the carbon chain does not result in increase in the affinity of ligand molecule for the metal ion as anticipated by Monk[16].

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R E S U M E

With the advent of newer physico-chemical techniques, and great theoretical advancement made by linderstrom-Lang, Cannan et al, Scatchard and Tanford regarding the electrochemical nature of the protein molecule, the problem of protein interaction has been successfully treated by a number of workers. Of the large number of possible protein interactions, the one related to the metals are of particular importance since a precise knowledge of the metal ion binding capacities of proteins, as well as their mode of interaction (depending on the availability of reactive sites in different pH media) is essential in solving the intricacies of the complicated metabolic processes in human system and in many other problems pertaining to industry and technology. Results of far reaching importance have been achieved with simple globular proteins. A summarised statement of the work on serum albumin from three different groups of investigators, which illustrate the mode of attack on the problem is worthmentioning.

(i) Klotz and Co-workers, mainly employed the spectrophotometric technique to investigate the nature of cupric ion binding to bovine serum albumin. Their study, particularly in the lower pH range, may be taken as an excellent example of metal ion binding to the carboxyl

R E S U M E

groups of protein. They have also shown that CO-NH_2 linkage become possible in this protein at alkaline side of pH.

(ii) Polarographic studies by Tanford, on the interaction of Cu, Zn, Cd, Pb, and Tl with bovine serum albumin threw further light on the nature and mode of metal-protein interaction. Considering the decrease in diffusion current of metal ions, in presence of protein as a function of pH, Tanford was able to calculate the extent of metal-protein binding and corresponding intrinsic association constants. The order of complexing tendencies with the imidazole groups of bovine serum albumin was found to decrease in the direction (log K values given in parentheses)

$$Cu(3.7) > Zn(2.9) > Cd(2.8) > Pb(2.3) > Tl(\geq 0)$$

(iii) Gurd and co-workers, on the basis of equilibrium dialysis studies have provided evidence of the binding of Zn with imidazole groups and Pb with carboxyl groups of serum albumin. The mechanism put forwarded by them necessitated the competition between the metal and hydrogen ion for a common site resulting in a decrease in the pH of the protein solution. In fact, such a behaviour was observed by Gurd and Goodman, who found that the addition of zinc chloride to isoionic serum albumin caused a detectable shift in pH.

The above mentioned studies, although carry much wealth of informations, however, are mainly limited to simple globular protein and speak very little about the quantitative aspect of the interaction of metal ions with fibrous protein. Extensive studies in this direction were therefore planned for the first time in this laboratory by Malik and co-workers (Ref. No. 4, 6, page 75-76) employing transfusion gelatin. Another new and interesting aspect investigated by these workers was to determine the availability of metal ions from their hydrous oxide sols for interaction with the protein (Ref. No. 24-27, page 205), which according to them was likely to prove of much physiological importance in course of time. The resume of the present work given in the following pages is an extension and advancement of their work.

1. - Part I of the thesis deals with the quantitative studies on the binding of Pb(ii), Sn(ii), Cu(ii), Cd(ii), Zn(ii), Co(ii) and Ni(ii) to transfusion gelatin carried out with the help of polarographic, pH-metric and equilibrium dialysis techniques.

The behaviour of transfusion gelatin complexes of Pb(ii) and Sn(ii) can be very satisfactorily studied at dropping mercury electrode. The conventional polarographic equation for complex metal ion, based on the shifts in half wave potential could not be used here. Tanford's method with all its limitation was found to be suitable and yielded useful results. The binding data were calculated

(b) The amount with which a particular metal ion displaces the hydrogen ion equilibria of transfusion gelatin towards the basic side of the functional groups, may be taken as a measure of the extent of metal-protein combination; and on further assuming with Gurd and Murray (Ref. No. 63, page 79) that one-to-one binding is favoured in preference to interamolecular cross linking than the number of hydrogen ions displaced per protein molecule, directly yields the binding data ($\bar{V}_M$).

Intrinsic association constants were calculated from $\bar{V}_M$, thus obtained. Lead, cobalt, nickel and cadmium appear to combine preferentially with the carboxyl groups, whereas copper and zinc both have got strong binding tendency with imidazole groups. In the latter case, however, the possibility of interaction with carboxyl of the protein in lower pH range is not ruled out. These observations are further supported by the direct measurements of binding data by equilibrium dialysis technique. The relevant intrinsic association constants obtained by different methods are summarised below

Intrinsic Association Constants for metal-carboxyl group linkage

Method	Log K-values						
	Cu	Zn	Cd	Co	Ni	pb	Sn
Polarographic	-	-	-	-	-	1.87	1.94
pH-metry	2.1	1.87	1.76	2.02	1.86	2.00	-
Equilibrium dialysis	2.2	1.87	1.96	-	-	-	-

employing Tanford's equation:

$$\frac{id}{(id)_o} = \frac{C_F + KC_b}{Co}$$

and intrinsic association constants were calculated using well known Scatchard relationship

$$K = \frac{V_M}{(n-V_H-V_M)\ C_F}$$

Experiments carried out with varying concentration of reactants at a fixed ionic strength and under varying pH conditions have shown the existence of a metal-protein complex in the pH range 3.7 to 5.5. This again suggested that the carboxyl groups offer themselves as principal sites for metal ion binding.

pH-metric studies on the systems like Pb-transfusion gelatin, Zn-transfusion gelatin, Co-transfusion gelatin, Cu-transfusion gelatin, Cd-transfusion gelatin and Ni-transfusion gelatin provided results of still greater interest. The hydrogen ion titration curves of transfusion gelatin in presence and absence of metal ions were strikingly different. Assuming that the metal ions compete with the hydrogen ions for common site, the following condlusions were arrived at.

(a) The amount of hydrogen ions given out by the protein are greater in presence of metal ions than with the protein alone. This would be the case if replacement of hydrogen ions by metal is visualised from the site under consideration.

Intrinsic Association Constant for metal-imidazole group linkage

Method	Log K-values	
	Cu	Zn
pH-metry	3.3	2.76
Equilibrium dialysis	3.4	2.91

(It should be noted that the constancy in log K-values obtained by polarographic, pH-metric and equilibrium dialysis methods, suggest that the assumption inherent in the calculation of pH-metric results are rather significant).

2. - Spectrophotometric studies on the binding of Cr(iii) to transfusion gelatin and bovine serum albumin, along with the biuret reaction of Cu and Ni-transfusion gelatin are described in the second part of the thesis. The main aim of these studies was to reinvestigate the problem of chromic ion fixation, employing a simpler and better characterised protein. Experiments carried out between pH 3.7 to 5.8 with changing metal protein ratio, indicate the binding of metal with the carboxyl groups of both the proteins. The close similarity between the spectra of Cr-transfusion gelatin (580 mu), Cr-bovine serum albumin (580 mu) and Cr-propionic acid (580 mu) systems further supported the above contention. The extent of metal ion binding was found to be dependent upon metal-protein ratio. The presence of a large proportion of metal ions in the reaction mixture brings about a decrease in the binding capacity.

On the other hand, the presence of large amounts of protein invariably brings about an increase in the binding of metal to the protein. The effect of the neutral salt and anions of the buffer solution on the metal-protein interaction is also interesting. The extent of metal-protein combination incfeases with increase in the concentration of neutral salt (KCl), whereas the presence of acetate ions seems to exert a negative influence. This effect may be explained in the light of Gustavson's assumption, that the chromic ions are rendered less reactive due to the formation of uncharged chloro-complexes $(Cr_2(OH)_2Cl)^{\circ}$ in acetate medium (Ref. No. 36 page 174).

One of the most interesting features of the chromic ion binding to both the proteins is that the transfusion gelatin with its lesser number of carboxyl groups (84) in comparison to bovine serum albumin (100) has got greater binding capacity. These observation lead to one to conclude that, collagen type of proteins with their peptide chains more or less extended and oriented in parallel pattern may form an intra-or intermicellar corss linking. This type of linkage as demonstrated by Gustavson (Ref. No. 37, page 174) contains several chromium atoms by means of two or more carboxyl groups of adjacent protein chains. In fact Kuntzel (Ref. No. 38, page 174) have shown that intramicellarly combination occured between collagen and vegetable tannino. On the other hand globular proteins have a compact and folded structure, and such a multipoint combination may not be feasible in case of serum albumin.

Studies on biuret reaction of transfusion gelatin with copper and nickel provide some valuable informations. Copper forms two complexes namely red and violet, which absorb maximum light at 525 mu and 550 mu respectively, whereas nickel forms only one complex corresponding to violet complex of copper, which absorb maximum light at 430 mu. Strikingly enough a number of bivalent metal ions have shown characteristic effect upon the 430 mu peak of nickel transfusion gelatin complex. Copper completely replaced nickel, whereas cobalt, zinc, cadmium and lead have got much less influence. The extent with which the different bivalent metals reduce the absorption peak of nickel-transfusion gelatin complex may be taken as a measure of the relative affinities of these metal ions for protein under biuret conditions. The following order is noted:

$$Cu > Ni > Co > Cd = Zn > Pb$$

3. - The interaction of metals and their hydrous oxide sols with more complicated proteins (egg albumin and casein) was also studied to explore the possibility of metal (both from the sol and electrolyte) entering into combination with the available sites on the protein molecule. pH-metry and viscosity methods were employed. The following informations were forthcoming on the basis of these studies.

(i) - pH-titration and viscosity-concentration curves show that chromium and aluminium (both from sol and electrolyte) make themselves available for combination with the protein.

The binding was found to be effected through carboxyl groups of casein and egg albumin. Chromium has got the greater affinity as compared to aluminium for both the proteins, whereas casein binds more metal ions than egg albumin.

(ii) - Iron appears to combine with the amino groups of casein and egg albumin. The nature of the titration (flat portion occuring in the pH range 10.5 - 9) as well as inflexion in the viscosity-concentration curve further indicate; that the OH groups (from tyrosine residue) probably play a significant role in ferric ion binding.

(iii)- Cobalt and nickel, both show the characteristic binding in the pH range 9 - 9.5. This indicates that amino groups of egg albumin offer themselves as principal sites for cobalt and nickel binding.

4. - The last part of the thesis deals with the studies on the interaction of metal with amino acids and peptide. These studies are significant in as far as that, the data on metal-small molecule interaction help in understanding the role of metal ions in more complicated systems. Attempt has been made to calculate the stepwise and over-all stability constants of various lead- amino acid, Chromium - amino acid and chromium-glycylglycine complexes, employing Bjerrum's method as illustrated by the work of Albert (Ref. No. 13, page 228).

The formation curves were obtained by plotting log(A) against $\bar{n}$. These curves indicate that the various amino acids used form stepwise complexes. The values of log (A) at $\bar{n}$ = 1 were taken from these curves and the over all stability constants were calculated using the equation.

$$\log K_s = -2 \log (A)$$

It has been found that the stability constant of various Pb-amino acid complexes fall between Zn. and Co-amino acid complexes (vide Albert Ref. No. 13, page 228). The order of complexing tendencies of bivalent metal ions for a series of amino acids (Reported by Monk Ref. No. 16, page 228 and Albert Ref. No. 13, page 228) Ni $>$ Zn $>$ Pb $>$ Co. is thus confirmed. Another interesting feature of these studies is that the difference in overall stability constants for various lead and chromium complexes is not strikingly large. These observations lead to conclude that the metal-amino acid linkage in various amino acids may not be radically different. However proline appears to have greatest affinity for both, lead and chromium. The order of affinity for lead is as follows (log Ks values are given in parentheses).

Proline (10.1) $>$ Glycine (9.2) $>$ Hydroxy proline (9.1) $>$ Asparagine (8.28) $>$ Lysine (8.18) $>$ Valine (8.2) $>$ Threonine (7.52) $>$ Phenyl alanine (7.4) $>$ Serine (7.26) $>$ Melhionine (7.14).

and for chromium

Proline (16.3) > Glycine (13.7) > Hydroxy proline (13.6) > Threonine (13.1) > Phenyl alanine (13.0) > Glycylglycine (10.3).

The following generalisations may be derived on the basis of above order.

(i) - The increase in length of carbon chain greatly decreases the stability constant of the complexes.

(ii) - The large and bulky  group at the α-carbon atom exert somewhat negative influence on complex formation.

(iii) The introduction of OH group in the chain has no marked effect on complex formation. In fact OH group should bring about an increase in the affinity as anticipated by Monk.

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POLAROGRAPHIC AND pH-METRIC STUDIES ON THE INTERACTION OF LEAD WITH TRANSFUSION GELATIN

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Very little is known regarding the binding of heavy metal ions to gelatin. The process can be better investigated by using a simple and well characterised gelatin like transfusion gelatin[1] and the first work of this kind was undertaken in this laboratory, when many interesting results on the binding of hydrogen ion[2] and heavy metal ions[3,4] to this particular protein were obtained. The behaviour of the Cu(II) complex at the dropping mercury electrode was particularly interesting[3] and it was, therefore, thought worthwhile to extend the studies to other metal ions. This paper deals with the polarography of lead ion in the presence of transfusion gelatin. Results on pH-metry have also been included to supplement the polarographic results.

A few valuable references on lead-protein binding are available in the literature. These are: (i) the work of TANFORD[5] on the binding of Pb(II) to bovine serum albumin (ii) polarographic estimation of lead in the presence of gelatin[6]; (iii) the investigation of GURD AND MURRAY[7] on the combination of lead ion with human serum albumin by the equilibrium dialysis technique and (iv) an indirect approach to the study of the lead complexes of egg albumin, haemoglobin and gelatin by SUZUTANI[8,9] who estimated free lead polarographically in the solution obtained after the complete adsorption of the complexes on animal charcoal.

EXPERIMENTAL

Transfusion gelatin (concn. 6%, mol.wt. = 75,000) supplied by the Director, N.C.L., Poona, was used throughout these investigations. Solution of lead nitrate and potassium nitrate were prepared by dissolving the appropriate A.R. salt in doubly distilled water. Walpole acetate and ammonium acetate buffers were prepared in the laboratory and their pH determined by the Beckman pH Meter, Model G.

Polarographic measurements were carried out by means of the Fisher Electropode in conjunction with the Multiflux Galvanometer (Type MG F2) in the external circuit. An H-shaped polarographic cell was found to be suitable for de-areation of the protein solution and for subsequent measurements at the D.M.E. The capillary used had a flow rate of 2.2 mg/sec with a drop time of 3.6–3.8 sec. The temperature of the solution was maintained at $30 \pm 0.1°$ by keeping the cell immersed in a thermostatic water bath (Townson and Mercer, Croydon).

The following sets were subjected to polarographic analysis:

(i) 1 ml of lead nitrate solution (0.01 *M*), 8 ml of acetate buffers pH 3.72, 4.45, 4.8,

5.2 and 5.57 and 2 ml of protein solutions were mixed and the total volume made up to 20 ml by adding potassium nitrate so that the ionic strength was adjusted to 0.15 in each case. Similar sets were analysed, using ammonium acetate–ammonia buffers of pH 5.5, 5.95, 6.35, and 6.8. The diffusion current of lead ions at different pH values in the absence of protein remained constant up to pH 5.5 and then decreased with increase in pH. This was attributed to the existence of aqua-complexes[5] at higher pH ranges. This fact was taken into consideration when computing the value of $i_d/(i_d)_0$. The results are shown in Table 1 (A and B) and Fig. 1.

TABLE 1A

CONCENTRATION OF PROTEIN, $0.8 \cdot 10^{-4}$ M; CONCENTRATION OF Pb(II), $0.5 \cdot 10^{-3}$ M; WALPOLE ACETATE BUFFER

pH	$i_d/(i_d)_0$	$C_b \cdot 10^{-3}$ M	$\bar{V}_M$
3.72	0.918	0.136	1.7
4.45	0.910	0.150	1.9
4.80	0.902	0.163	2.0
5.20	0.869	0.218	2.7
5.57	0.853	0.245	3.1

TABLE 1B

CONCENTRATION OF PROTEIN, $0.8 \cdot 10^{-4}$ M; CONCENTRATION OF Pb(II), $0.4 \cdot 10^{-3}$ M; AMMONIUM ACETATE–AMMONIA BUFFER

pH	$i_d/(i_d)_0$	$C_b \cdot 10^{-3}$ M	$\bar{V}_M$
5.50	0.810	0.253	3.2
5.95	0.790	0.280	3.5
6.35	0.780	0.293	3.6
6.80	0.780	0.293	3.6

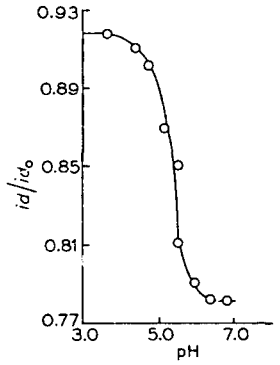

Fig. 1. Effect of pH on the diffusion current depression; $\mu = 0.15$.

(ii) Varying amounts of protein were mixed with a fixed amount of lead nitrate (1 ml) and acetate buffer (8 ml of pH 5.57) was added and the total volume made up to 20 ml with the ionic strength being kept at 0.15 in each case. Solutions of high protein content ($1.8–2.0 \cdot 10^{-4}$ M) precipitated within an hour. In these cases analysis was carried out immediately after mixing. The results are shown in Table 2 and Fig. 2.

TABLE 2

CONCENTRATION OF Pb(II), $0.5 \cdot 10^{-3}$ *M*

Concn. of protein $\cdot 10^{-4}$ *M*	$i_d/(i_d)_0$	$C_b \cdot 10^{-3}$ *M*	$\overline{V}_M$
0.4	0.914	0.143	3.5
0.6	0.885	0.191	3.2
0.8	0.853	0.245	3.1
1.2	0.814	0.310	2.6
1.4	0.743	0.428	3.0
1.6	0.728	0.453	2.8
1.8	0.709	0.485	2.7
2.0	0.704	0.493	2.5

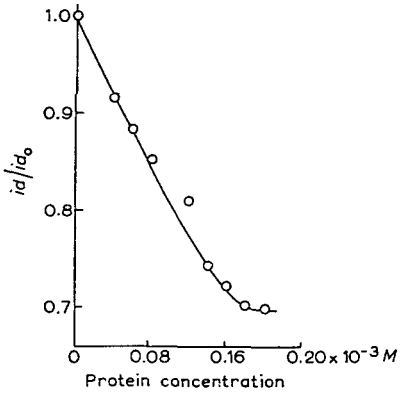

Fig. 2. Effect of protein concn on the diffusion current decrease.

TABLE 3

CONCENTRATION OF PROTEIN, $0.8 \cdot 10^{-4}$ *M*

Concn. of Pb(II) $\cdot 10^{-3}$ *M*	$i_d/(i_d)_0$	$C_b \cdot 10^{-3}$ *M*	$\overline{V}_M$
1.0	0.893	0.356	4.4
0.75	0.866	0.335	4.1
0.50	0.853	0.245	3.1
0.35	0.840	0.186	2.3
0.20	0.725	0.183	2.2
0.10	0.700	0.100	1.2

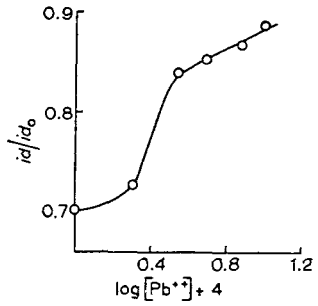

Fig. 3. Effect of metal ion concn. on diffusion current decrease.

(iii) Varying amounts of lead nitrate solution were mixed with a fixed amount of gelatin, and acetate buffer (8 ml of pH 5.57) was added keeping the total volume 20 ml and ionic strength at 0.15. The results are summarised in Table 3 and Fig. 3.

In all, 70 polarograms were taken. The typical ones are illustrated in Fig. 4. The reversibility was tested by the equation given by TOMES[10].

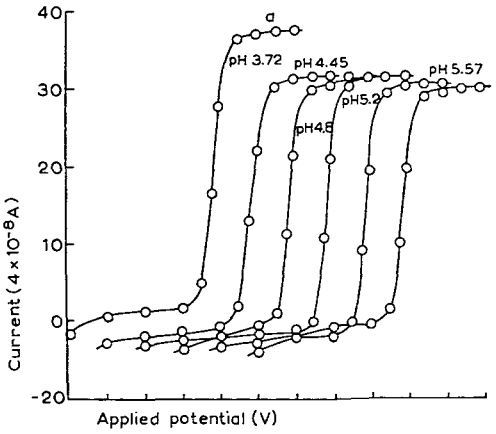

Fig. 4. Polarograms. Protein concn. $0.8 \cdot 10^{-4}$ M; Pb^{2+} concn. $0.5 \cdot 10^{-3}$ M; $\mu = 0.15$ Curve (a), protein absent. Each curve starts at 0 V, 1 cm = − 0.1 V.

The following method was adopted for pH-metry:

2 ml of transfusion gelatin (3%, pH 5.8), 2 ml of lead nitrate solution (pH 5.8) and varying amounts of hydrochloric acid were mixed in different test tubes keeping the total volume at 10 ml and the ionic strength at 0.15. The pH of each solution was recorded immediately after mixing and then again after 24 hours. The value were found to be the same in both cases. The measurements were repeated without lead ion but maintaining the same conditions. The results are given in Table 4.

RESULTS AND DISCUSSION

Taking the molecular weight of transfusion gelatin as 75,000, the number of metal ions bound per protein molecule, $\bar{V}_M$, was computed from the values obtained by the following relationship:

$$C_b = C - C_F$$

and TANFORD's equation

$$i_d/(i_d)_0 = \frac{C_F + kC_b}{C}$$

where C_F, C_b and C are the free, bound and total metal concentration; $id_i'(id)_0$ is the depression in diffusion current (given in column 2 of Tables 1, 2 and 3); k is the limiting value of $i_d(i_d)_0$ obtained from curves 2 and 3.

The values given in Table 4, column 2, are converted into free hydrogen ions assuming that the activity of hydrogen ion (a_{H^+}) depends only upon the non-protein constituent of the solution even in presence of protein. The pH is given as

$$pH = -\log a_{H^+}$$

The activity coefficients at ionic strength 0.15 (in extreme acid ranges) are taken from TANFORD[11]. The difference between added and free hydrogen ions gives the number of hydrogen ions bound to the protein (Table 4, column 4), From this the number of hydrogen ions dissociated per protein molecule is evaluated both in the presence and absence of metal ions (Table 4, column 5).

TABLE 4A

CONCENTRATION OF PROTEIN, $0.8 \cdot 10^{-4}$ *M*

H⁺ added (moles/l·10⁻³)	*pH*	*Free H⁺ (moles/l·10⁻³)*	*Bound H⁺ (moles/mole protein)*	*Moles of H⁺ dissociated per mole protein*
0	5.56	—	—	—
0.8781	4.9	0.01258	11.0	59
1.7562	4.6	0.02511	22.0	48
3.5124	4.0	0.1000	43.0	27
5.2686	3.5	0.3162	62.0	8
6.1467	3.15	0.7029	68.0	2
7.9029	2.7	2.223	71.0	0
12.2936	2.23	6.683	70.0	0

TABLE 4B

CONCENTRATION OF PROTEIN, $0.8 \cdot 10^{-4}$ *M*; CONCENTRATION OF Pb(II), $0.4 \cdot 10^{-3}$ *M*

H⁺ added (moles/l·10³)	*pH*	*Free H⁺ (moles/l·10³)*	*Bound H⁺ (moles/mole protein)*	*Moles of H⁺ dissociated per mole protein*
0	5.58	—	—	—
0.70248	4.7	0.01995	9.0	61
1.7562	4.3	0.05011	21.0	49
3.5124	3.7	0.1995	41.0	29
5.2686	3.3	0.5011	59.5	10
6.1467	3.08	0.8709	66.0	4
7.9029	2.65	2.494	67.0	3
12.2936	2.22	6.837	68.0	2

The appreciable reduction in diffusion current of metal ions in the presence of a large amount of protein has been ascribed to: (i) the probable complex formation[5,12,13,14] between metal and protein; (ii) adsorption[15] and (iii) viscosity[12] effects. Since the present investigations were carried out at pH 5.5 and above, and every care was taken to keep the protein in the native state, the influence of non-specific factors like adsorption and viscosity can very well be ruled out, and the relative depression in diffusion current can be safely taken as a measure of metal–protein combination. The fact that the value of $\bar{\nu}_M$, the number of metal ions bound per protein molecule, remained the same at all protein concentrations, and a limiting value for $i_d/(i_d)_0$ was realised either by changing the metal: protein ratio or pH, also leads to the same conclusion.

From Table 1 it is seen that C_b is highly dependent upon pH. The changes are most marked in the vicinity of pH 5.0, showing therefore that the uptake of metal ions by

the protein increases more rapidly in the pH range 5.2–5.57. Here all the 84 carboxyl groups of transfusion gelatin are deprotonated[2], and are thus available for the binding of metal ions. It may, therefore, be concluded that the carboxyl groups offer the principal sites for the binding of plumbous ions.

This view is further supported by the results on hydrogen ion equilibria in the presence and absence of metal ions (Table 4 A and B). Assuming that metal ions compete with hydrogen ions for the common site (carboxylate ion) on the protein molecule, the following conclusions may be drawn:

1. The amount of hydrogen ions given out by the protein is greater in the presence of lead than with protein alone (Table 4 A and B, column 3). This would be the case if the replacement of hydrogen ions by lead, from the carboxyl groups of the protein, is visualised, especially as the observations were made at the lower pH range.

2. There is a difference of two hydrogen ions per protein molecule for mixtures with and without metal ions (Table 4 A and B, column 5). Normally such data would point towards a possible combination of one lead ion with two carboxyl groups resulting an intramolecular cross-linking. But we assume, with GURD AND MURRAY[7], "a one-to-one binding", which appears to be more plausible in the case of the lead–protein complex[16]. This difference in hydrogen ion concentration directly determines the number of Pb(II) ions bound per protein molecule[17], thus $\bar{\nu}_M$ appears to be 2 at a total metal concentration $0.4 \cdot 10^{-3}$ M.

The intrinsic association constant for the lead–gelatin complex was calculated by applying SCATCHARD's equation.

$$K = \frac{\bar{\nu}_M}{(n - \bar{\nu}_H - \bar{\nu}_M)C}$$

where $\bar{\nu}_M$ and $\bar{\nu}_H$ are the active sites covered by the metal ions and hydrogen ions respectively, n is the total number of such sites, C is the total metal concentration and K is the intrinsic association constant, the values of n and $\bar{\nu}_H$ are taken from the literature[2]. Using this equation, log K was found to be 1.87 by inserting the value for $\bar{\nu}_M$ obtained by polarography, and 1.91 by inserting the value for $\bar{\nu}_M$ determined indirectly from pH-metry. The constancy of log K is proof of the assumption that only one Pb(II) ion is bound with one carboxyl group and that the pH-metric method is valid.

The free energy change of the combination at 30° was

$$\Delta F = -2.593 \text{ kcal.}$$

ACKNOWLEDGEMENT

Thanks are due to Prof. A. R. KIDWAI for providing facilities and to Prof. K. VENKATARAMAN, Director, National Chemical Laboratory, Poona, India for the supply of transfusion gelatin.

SUMMARY

Polarographic and pH-metric studies of mixtures containing lead nitrate and transfusion gelatin were undertaken to ascertain how plumbous ions were bound to the protein. Experiments carried out with varying concentrations of the reactants at a fixed ionic strength and under vaying pH conditions have shown the existence of a

metal–protein complex in the pH range 3.7–5.57. Experiments carried out at a higher pH range (5.9–6.8) gave little or no evidence for the binding of lead to the imidazole groups. An alternative procedure, identical in principle with BJERRUM's method, was employed to calculate $\bar{\nu}_M$ from the difference in hydrogen ion data in the presence and absence of metal. Evidence is presented for a one-to-one binding of plumbous ion to the carboxylate ion of transfusion gelatin, for which the intrinsic association constant was calculated as 1.87 and the free energy change of the combination as -2.593 kcal.

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Spectrophotometric Studies on the Binding of Cr(iii) to Transfusion Gelatin

By Wahid U. Malik and M. Muzaffaruddin

With 3 figures

Sumary

Spectrophotometric studies of mixtures containing Cr(iii) and transfusion gelatin were undertaken to know the binding of chromic ion to the protein. Experiments carried out between pH 3.7 and 5.8 had indicated the binding of the metal through the carboxyl groups of the protein. The extent of metal binding was found to be dependent upon metal-protein ratio, maximum binding taking place in the ratio metal to protein as 42:1.

The nature of metal-protein binding has been successfully worked out by a number of workers[1–10]), using spectrophotometric method. Investigations in this direction have, however, been less extensive with chromium as the metal bound. References worth mentioning are (i) KÜNTZEL's[11-12]) work on the chromium glycine complex to demonstrate the role of chromic ions in chrometanning, (ii) GUSTAVSON's[13])[14]) researches on chromium-pro-

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tein interaction leading to the conclusion that the metal ion got bound through carboxylic groups of proteins. Since the above mentioned studies were limited to gelatin, it was thought worthwhile to reinvestigate the problem systematically employing more simpler species. In the present communication the influences of factors, viz. concentration of the reactants, pH and ionic strength on the extent of binding of chromium to transfusion gelatin are discussed.

Experimental

Reagents

Transfusion gelatin (6 per cent Con.) was used throughout the investigation. Chromic chloride (BAKER, A. R.) potassium chloride (A. R.) were used for preparing the solutions, chromium concentration was determined colorimetrically[15]. Walpole acetate buffers were prepared from 0.2 M solutions of acetic acid and sodium acetate and their pH's were checked by BACKMAN pH meter Model G using glass electrode.

Apparatus

Light absorption measurements were carried out by Model DU spectrophotometer using tungsten lamp as the light source and corex cell (1 cm. depth).

Procedure

The following sets were analysed.

(i) Chromic chloride (1×10^{-2} M) and gelatin (1.8 per cent) were mixed in a number of pyrex boiling tubes. Their pHs were adjusted to 3.7, 4.4, 4.8, 5.2, 5.57 and 5.8 by the addition of buffers. The ionic strength was maintained at 0.4 by adding requisite amount of 1 M KCl. Another set in which the pHs were adjusted by the addition of dilute KOH instead of the buffers was also analysed spectrophotometrically.

(ii) At a fixed pH (5.57) and ionic strength (0.4) two sets of mixtures were prepared. In one the chromium concentration (1×10^{-2} M) was kept fixed and the concentration of the protein was varied while in the other there was fixed amount of protein and the concentration of the metal ion was changed.

(iii) Mixtures were analysed spectrophotometrically at three different ionic strength Viz. 0.5, 0.4, and 0.2 taking varying concentrations of metal ion with a fixed amount of protein (1.8%).

The molar extinction coefficient E of chromic ion was calculated by means of the expression.

$$\text{Log } \frac{I_0}{I} = E\,Cd$$

where C is the molar concentration of chromium, d is the depth of the cell (1 cm.) and Log I_0/I is the observed optical density at $\lambda_{max} = 580$ mμ. The results are given in Table 1 and 2.

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Table 1
Effect of pH, metal and protein concentration on chromium transfusion gelatin interaction

(a) Protein con. 1.8%, $[Cr^{+++}] = 1 \times 10^{-2}$ M, $\mu = 0.40$						
(i) pH (with acetate buffer)	3.7	4.4	4.8	5.2	5.57	5.8
E-values (at 580 mμ)	24.2	25.4	27.7	29.0	30.00	30.4
(ii) pH (with KOH)	3.6	4.2	4.4	4.7	4.9	5.2
E-values (at 580 mμ)	32.5	33.5	34.5	35.4	36.5	37.2

(b) Protein conc. 1.5%, pH 5.57, $\mu = 0.40$							
Chromium con. ($\times 10^{-2}$ M)	0.25	0.50	0.75	1.0	1.5	2.0	3.0
E-values (at 580 mμ)	56.0	33.0	26.8	24.0	21.0	19.8	18.1

(c) Chromium con. 1×10^{-2} M, pH 5.57, $\mu = 0.40$								
Protein con. (%)	0.6	0.9	1.2	1.5	1.8	2.1	3.5	4.0
E-values (at 580 mμ)	16.2	17.7	20.8	24.0	26.0	30.2	33.0	35.2

Table 2
Effect of ionic strength on Chromium transfusion gelatin interaction
(a) Protein con. 1.8%, $\lambda_{max} = 580$ mμ

Chromium con. 1×10^{-2} M	E-values at ionic strenght		
	$\mu = 0.5$	$\mu = 0.4$	$\mu = 0.2$
0.5	48.0	41.2	39.6
1.0	32.5	31.6	31.5
2.5	—	18.6	18.3
3.0	20.1	20.3	20.5
4.0	19.0	19.2	19.5

Discussion

The absorption studies carried out in the pH range 3.7 to 5.8 at different wave lengths give a maximum at 580 mμ (Fig. 1) showing thereby the binding of the metal through the carboxyl groups of the protein[16]) (pK = 4.8) only. Moreover, since no shift in maximum is observed even at pH 5.8, it may be concluded that the imidazole groups of the protein are not involved in binding chromic ions (expected λ_{max} 540 mμ. GREEN and ANG[15]) on Cr(iii) alanine complex).

The molar extinction coefficient values at 580 mμ when plotted against pH give an S-shaped curve (Fig. 2, A and B). The inflexion occuring near about pH 5.0. This result again indicates that a large number of carboxyl

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groups are made available in this pH range (84 groups)[17]) and offer the maximum number of sites for the binding of chromium. A comparison of extinction coefficient values measured in acetate buffers and KOH also gives a few interesting results. It is found that E-values are larger in KOH medium than in the acetate buffers (Table I, a). From these results it may be

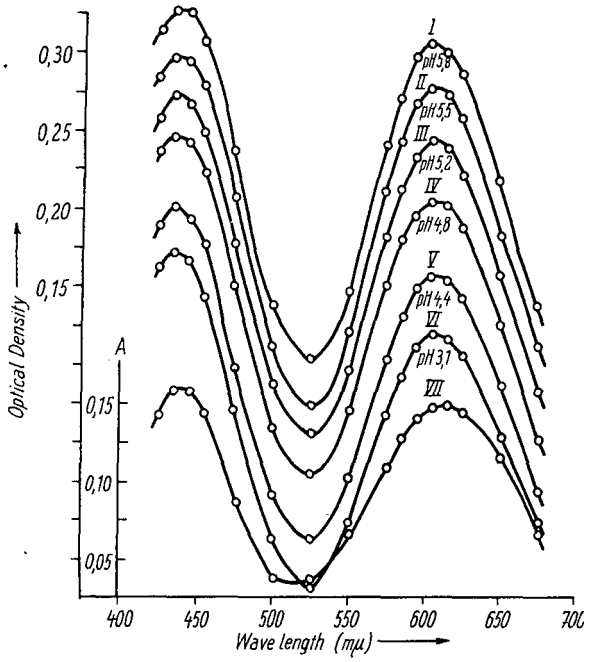

Fig. 1. Absorption spectra of Chromium-gelatin complex. Cuvers; VII for 1×10^{-2} M Cr^{3+}; I to VI for Chromium-gelatin mixtmes at different pHs. Scale A for curve VII

concluded that acetate ions exert a negative influence on metal protein interaction. Our results may be interpreted in the light of GUSTAVSON's[18]) assumption who visualises a lesser reactivity of the chromium ions in presence of the acetate solution due to the formation of uncharged chloro-species $(Cr_2(OH)_5Cl)^\circ$ of the chromic ions.

Metal protein ratio appears to exert a large influence on the binding of chromium to the protein. It is evident from the results on the spectrophotometric titrations carried out with metal ions in presence of a fixed amount of protein and vice versa (Table 1, b and c). From the results it may be conc-

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luded that the presence of a large proportion of metal in the reaction mixture brings about a decrease in the binding capacity. On the other hand, presence of larger amounts of protein in the reaction mixture invariably brings about an increase in the binding of the metal to protein. On plotting the difference of optical density of the metal ion and the complex against

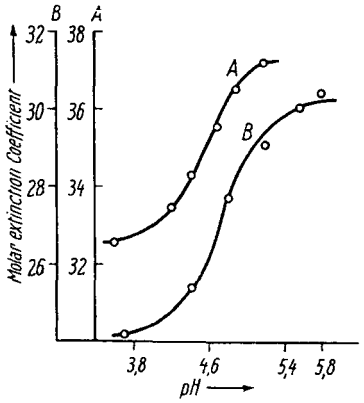

Fig. 2. Effect of pH on molar extinction coefficent. Curve A with KOH and Curve B with acetate buffer: Protein concentration $1 \cdot 8\%$ and concentration of Cr^{3+}, 1×10^{-2} M

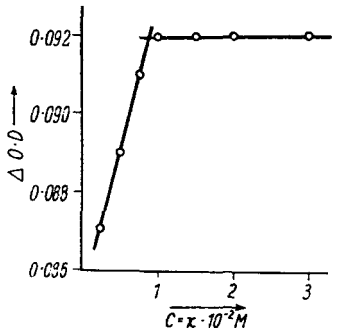

Fig. 3. Plot of $\triangle$ O · D (difference in optical densities of chromium-gelatin mixture and that of chromium alone) against C (chromium concentration)

metal ion concentration an inflexion is observed at metal concentration equal to 0.85×10^{-2} M (Fig. 3). This interesting observation leads us to conclude that the maximum binding takes place at the metal protein ratio $0.85 \times 10^{-2} : 0.2 \times 10^{-3}$ that is 42 : 1.

Experiments performed at three ionic strengths, viz. 0.2, 0.4 and 0.5 go to show that the extent of metal protein interaction increases with increase in ionic strength. The effect is most pronounced in dilute solution rather than in concentrated one where almost no change in extinction coefficient values is observed with increase in the ionic strength (Table 2).

Thanks are due to Professor A. R. KIDWAI for providing facilities and to Professoer VENKATARAMAN, Director, National Chemical Laboratories, Poona, India for the supply of transfusion gelatin.

Aligarh, Chemical Laboratories, Aligarh Muslim University.

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Spectrophotometric and Potentiometric Studies on the Composition and Stability of Chromium Propionate Complex

Mohd. Muzaffaruddin, Salahuddin and Wahid U. Malik

Spectrophotometric and conductometric methods show the existence of 1:1 complex for the product obtained by refluxing chromic chloride and propionic acid at 70° for 2½ hours. Bjerrum's method has been applied to determine the values of log K and $\triangle F$ of the complex, the values being 5.74 and −3.797 kcals. respectively.

The physico-chemical investigations regarding the interaction of Cr (III) with organic acids[1-3] are of great importance since these complexes have recently found wide applications in industry as coating materials[4]. Moreover, the complexity of the process of irreversible fixation of cationic chromium complexes through the carboxyl groups of collagen[5] in chrome-tanning further demands, for its proper interpretation, the undertaking of such studies.

Except the preliminary studies of Weinland[6], who obtained a green complex from CrO_3 and propionic acid and a violet complex from sodium propionate and chromic chloride, no systematic study has been described in the existing literature regarding the combination of chromium with propionic acid.

The present communication describes the detailed study of the interaction of chromium(III) with propionic acid, using spectrophotometric (modified molar-ratio[7] and Job's method[8,9] of continued variation) and potentiometric methods (Bjerrum's method[10]).

EXPERIMENTAL

A.R. samples of Cr(III) chloride and sulphate were used for preparing the solutions and their concentrations determined colorimetrically[11]. Propionic acid and potassium chloride were also of A. R. grade. Triple-distilled water was employed to prepare the solutions. Walpole acetate buffers[12] were made from 0.2M solutions and their pH's tested by a Beckman pH meter, Model G.

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Optical density measurements were carried out at 580mμ by means of a Beckman DU spectrophotometer, using tunsgten lamp as the light source and 1 cm corex cell. Lambert-Beer's law was found to hold good throughout the whole range of chromium concentration. The reaction between Cr(III) and propionic acid, being a slow one at room temperature, was therefore investigated at higher temperatures. Preliminary experiments were performed after heating the reaction mixture between 60° and 70° for some time.

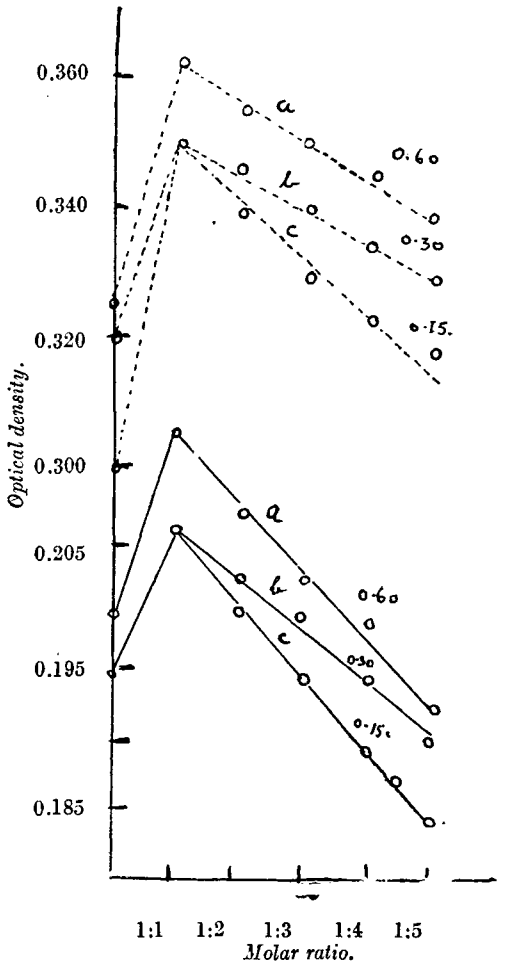

FIG. 1. Effect of ionic strength on Cr-propionate complex at *p*H 5.5.
The broken lines for 10×10^{-3} Cr^{3+} and full lines for 5×10^{-3} Cr^{3+}.

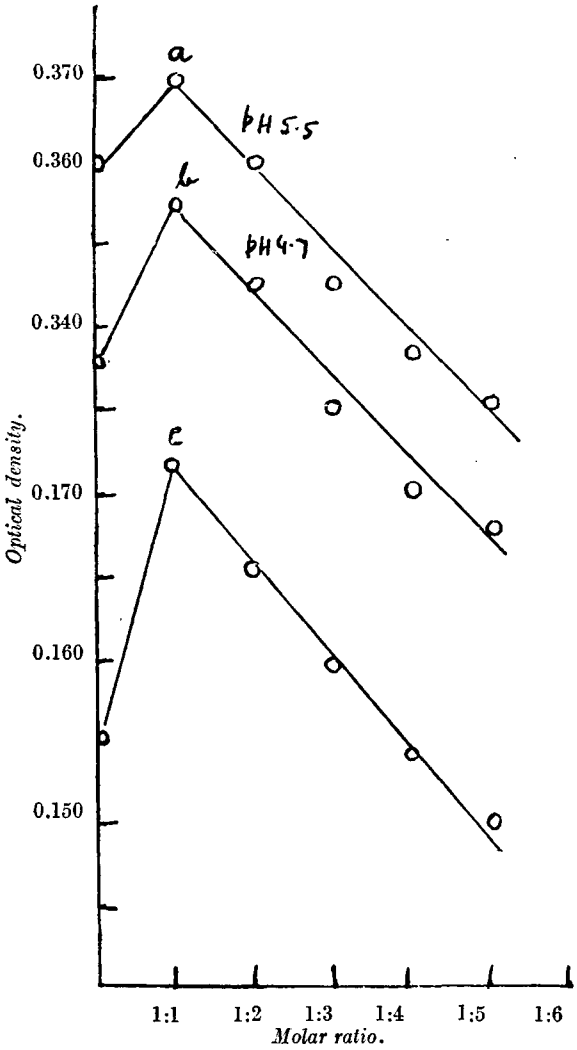

FIG. 2. Effect of *p*H on the spectra of the complex.
Cr^{3+} conc. for curves *a* and *b* is $10\times10^{-3}M$ and $5\times10^{-3}M$ for curve *c* which represents spectra without buffer.

An aliquot of the mixture (10 ml) was pipetted after short intervals, cooled to room temperature (30°), and optical density measured at different wave lengths to determine the absorption maxima. Although the O. D. values increased with lapse of time, the absorption maxima were found to exist at 580 mμ. When the mixture had been refluxed for 2½ hours, a constancy in the O. D. values could be realised. This condition was then taken for the attainment of equilibrium. Subsequent measure-

ments (optical density, *p*H, and conductance) were carried out after cooling to room temperature the reaction product obtained on refluxing the reaction mixtures at 70° for 2½ hours.

Solutions of chromic chloride (5 and 10 m*M*) were taken with propionic acid in the ratio (chromic chloride: propionic acid) 1:0, 1:1, 1:2, 1:3, 1:4, and 1:5 at three different ionic strengths (adjusted to 0.15, 0.3, and 0.60 by addition of KCl). The *p*H's were maintained at 4.7 and 5.5 by adding requisite amounts of buffers. Observations were carried out with and without addition of buffers. The results are shown in Figs. 1 and 2 (curves *a*, *b*, and *c*). Experiments under similar conditions were performed using Job's method[8]. The differences between the optical densities of the mixtures and those of the metal ions have been plotted against the varying concentration of chromium ion (Fig. 3, curves *a* and *b*).

Conductometric measurements under identical conditions were carried out by a Cambridge conductivity bridge, type L, 350140, using a Cambridge conductivity cell. The difference in conductance of the mixture and that of the metal ion has been plotted against the increasing concentration of Cr(III) (Fig. 4). No detectable change in the results was observed by either of the above two methods when chromic chloride was replaced by chromic sulphate.

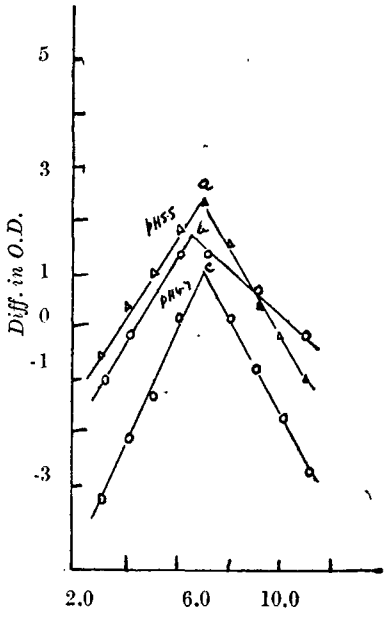

FIG. 3. Curve c represents the reaction mixture alone (*p*H <3).

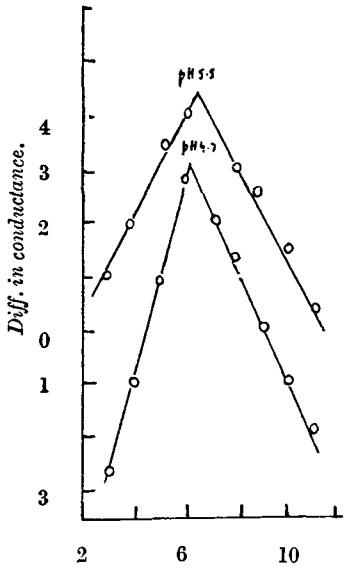

FIG 4.

*p*H *Titration* (*Bjerrum's Method*).—To the mixture (10 ml), containing the reactants approximately in the ratio 1:1, A. R. potassium chloride was added to adjust the ionic strength to 0.15. It was then refluxed for 2½ hours, cooled to 30°, and titrated with carbonate-free[13] potassium hydroxide (pure nitrogen was employed for inert atmosphere

13. Kolthoff and Sandell, "Text Book of Quantitative Inorganic Analysis", 3rd ed., The Macmillan Co., N. Y., 1952.

as well as for stirring). Column 6 of Table I records the value of log K at $\mu=0.15$ for the complex obtained at 70°.

Stability constant K at $\mu=0.15$ for the reaction:

$$M+C_2H_5COO \rightleftharpoons M(C_2H_5COO^-) \quad \ldots \quad (1)$$

was calculated with the help of the expression:

$$K = \frac{\bar{n}}{(1-\bar{n})\ (C_2H_5COO^-)} \quad \ldots \quad (2)$$

when $\bar{n}$, the average number of propionic acid molecule bound by one chromium atom, is given by

$$\bar{n} = \frac{C_A - \left[(C_2H_5COO^-)\right]\left[\frac{1+(H^+)}{K_a}\right]}{\text{Total concentration of Cr(III)}}$$

where C_A is the total concentration of propionic acid

(H^+) is known at a particular pH; the value of K_a is taken from the literature[15] The concentration of free complex-forming species $(C_2H_5COO^-)$ was evaluated with the help of the equation:

$$\log\ (C_2H_5COO^-) = (p\text{H}-pK_a + \log\ [C_A-(\text{KOH})]$$

Thus knowing the values of $(C_2H_5COO^-)$ at various pH's during titration, the value of $\bar{n}$, and hence the stability constant K, was calculated.

TABLE I

Potentiometric titrations.
Ionic strength=0.15. Temp.=30°.

[KOH].	pH.	$[C_2H_5COO^-]\times10^4$.	Log $[C_2H_5COO^-]$.	$\bar{n}$.	Log K.
0.00	2.88	..	..	..	..
10.65	3.22	2.01	4.3012	0.10	2.746
19.76	3.62	4.54	4.6571	0.20	2.740
31.75	9.02	9.64	4.9841	0.35	2.746
44.67	4.38	17.90	3.2529	0.50	2.740
63.02	4.92	41.50	3.6180	0.70	2.750
66.49	5.07	53.10	3.7251	0.75	2.750
71.95	5.27	70.40	3.8476	0.80	2.750

Mean 2.740

DISCUSSION

Job's method of continued variation provided a 1:1 complex for the chromic chloride-propionic acid reaction. The composition was not found to be influenced by the change in

14. See Ref. No. 4,
15. Everett *et al.*, *Proc. Roy. Soc.*, 1952, **A215**, 403.

*p*H (Fig. 3). The only influence of *p*H worth mentioning is that the absorbance is higher at *p*H 5.5 than at *p*H 4.7. From this it may be concluded that complex formation is favoured in the higher *p*H range.

The influence of ionic strength on the absorbance values is also worth considering. It was found that the optical density values did not show any marked change in the ionic strength range of 0.15 to 0.3. When it was increased to 0.6, an appreciable change from 0.35 to 0.362 was, however, observed (Fig. 1, curves *a* and *b*), but this is possibly not the only reason for such a behaviour. The increase in optical density may be due to the increase in the amount of the complex. Harvey and Manning[7] in their study of iron complexes of Tiron reported a similar phenomenon. Contrary to the theoretical point of view, their results showed a decrease in degree of dissociation of the complex instead of an increase at higher ionic strength.

Potentiometric titration of the complex reveals that more carboxyl groups of the acid are made available for binding with the metal as the *p*H of the reaction mixture is increased from 3.22 to 5.27 (studies beyond this *p*H could not be carried out because, unlike the complex, the metal ion was precipitated beyond *p*H 5.27). Such a behaviour is not unlikely in view of the high *pK* values of propionic acid (4.87). Here too, the combining ratio came out to be approximately 1:1. The value of log *K* was found to be 2.74 and that of $\triangle F^{\circ} = -3.797$ kcals.

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Polarographic Studies on the Interaction of Sn(II) with Transfusion Gelatin.

The identity of Sn(II) in many biochemical reactions connected with animal metabolism and enzyme activity[1,2] is well recognized, but their specific role in such reactions is not yet clear due to the lack of information on the binding of the metal with proteins[3]. An attempt was, therefore, made to work out the problem by carrying out polarographic studies with a well characterised protein like transfusion gelatin[4].

Mistures containing (i) fixed amounts of Sn(II) transfusion gelatin at varying pH (viz., 3.5 to 5.3); (ii) fixed amounts of Sn(II), varying amounts of protein at pH 5.0 and (iii) fixed amounts of protein, varying amounts of Sn(ii) at pH 5.0 were subjected to polarographic analysis Acetate buffer and potassium chloride were used to maintain the pH and ionic strength respectively, only the cathodic waves with a half wave potential 0.65 V vs S.C.E.[5] were considered. The experimental technique and method of calculations were essentially the same as given earlier[6a,7].

Discussion. The marked decrease in diffusion current of the metal in presence of increasing amount of the protein, coupled with the fact that a limiting value of $(id)/(id)_o$ (0.76 in the present case) is realized by changing the metal: protein ratio (Fig.1) indicates the uptake of metal ions by

the specific potential sites of the protein molecule[6a,7,8]. It is argued that factors like viscosity[8] and adsorption[9] may also be responsible for the decrease in the diffusion current. In the investigations under discussion such a possibility can be completely ruled out in view of the purely native state in which the protein is kept throughout experimentation and the little possibility of the negatively charged protein (pH 5) to be adsorbed on mercury drops. The values of $(id)/(id)_o$ obtained on increasing the pH from 3.5 to 5.3 also lead to the same conclusion.

The characteristic effect of pH on the extend of binding of Sn(II) to transfusion gelatin can very well be seen from the values of Vm, number of metal ions per protein molecule. The values range between 4 and 10 in the pH range 3.5 to 5.3 showing thereby that the carboxylic groups offer principal sites[6b] for the binding of tin. The intrinsic association constant calculated from SCATCHARD'S equation[10] comes out to be 89 when an average value of 9 is taken for VM at a total metal concentration 13.53×10^{-4} M. The value of the free energy change comes out to be: F = 2.708 Kcal.

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